Food Research

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SUGAR DISTRIBUTION IN PLANT TISSUES COOKED IN SYRUP[a,b]

CLARENCE STERLING AND C. O. CHICHESTER
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Davis, California

(Manuscript received February 17, 1959)

During a study *(3)* of the penetration of maltosaccharides into canned cling-stone peaches, distribution of the radioactive sugars was followed by using the method of autoradiography. According to that study, sugar penetration into the fruit occurs "in mass." However, higher concentrations of sugar were found in the vascular bundles than in the neighboring parenchymatous tissues.

Because of the intrinsic theoretical value, it was found desirable to investigate the precise cellular distribution of the radioactive sugars. It would be of interest to know if this distribution were random or localized.

MATERIALS AND METHODS

Microscope slides and nuclear track plates of an earlier investigation *(8)* were studied. In that investigation, fresh cling-stone peaches were peeled manually and cooked in a 30% syrup of glucose in distilled water. A small amount of the glucose was radioactive with carbon-14. Segments of the peaches were prepared for autoradiography, and nuclear track plates were exposed to thin sections of these segments *(8)*. In addition, the exposed plates and sections used in the study of sugar penetration by Hughes *et al (3)* were re-examined. Photomicrographs were made of the peach sections and their autoradiographs.

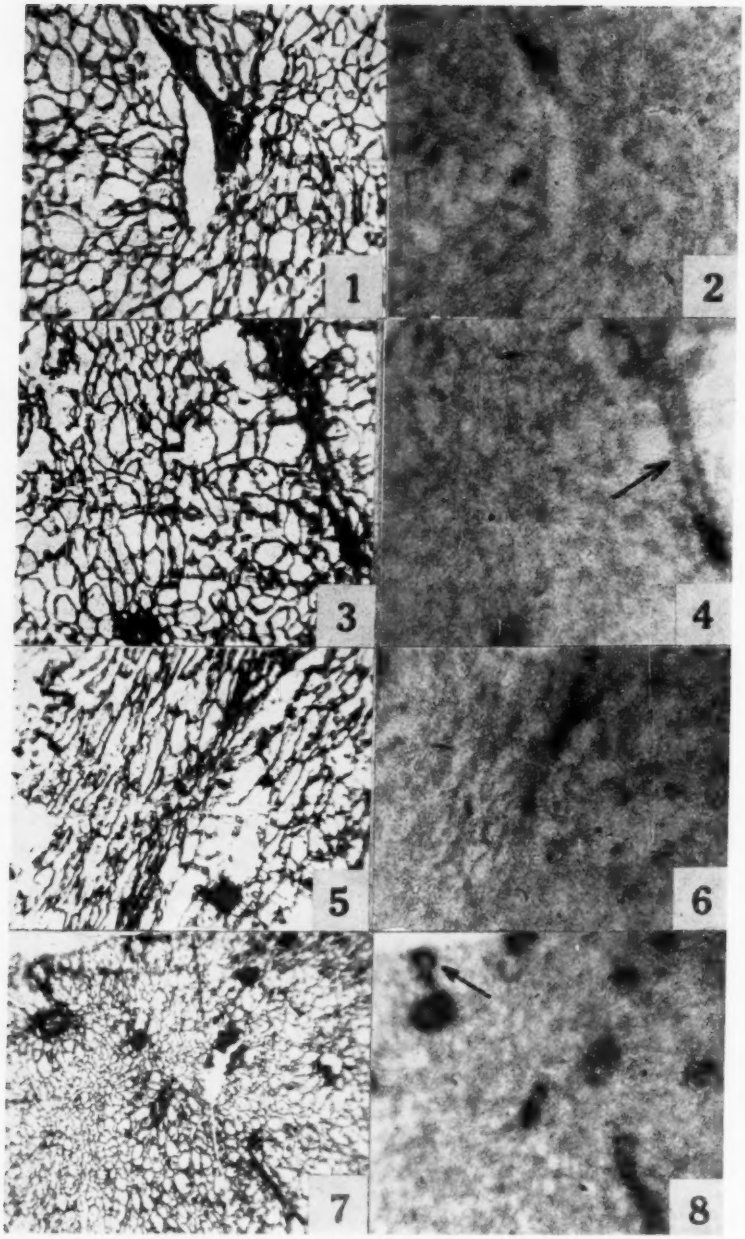
RESULTS AND DISCUSSION

When a close examination was made, it became apparent that cellular outlines were rather distinct on the autoradiographs (Figs. 2, 4, 6, 8) of the cooked peaches. Corresponding regions of the unstained peach sections are given in Figures 1, 3, 5, and 7. On careful inspection, a point to point agreement of cell configurations becomes obvious. Note that the cell lumen is much lighter than the cell wall in the autoradiographs. Note also that the intercellular spaces likewise show much lower radioactivity than do the cell walls.

Generally, the vascular bundles have the highest concentration of radioactive sugar. (The nuclear track plate of Figure 8 has been somewhat overexposed.) However, the sugar is not abundant in the lumina of the vascular cells. In the autoradiograph of the tracheary element in Figure 4 (at arrow), the far greater sugar concentration is in the cell wall. In Figure 8 (at arrow), there is shown the autoradiograph of an intercellular duct, which has been created by cellular lysis in the center of a vascular bundle. The duct is weakly radioactive. Nevertheless, the cell walls surrounding the duct are regions of strong radioactivity.

It could be supposed that sugar particles, derived from syrup in the cell lumen, might be deposited on interior cell surfaces during lyophilization

[a] This study was supported, in part, by a grant-in-aid from the American Maize-Products Company. The authors wish to express their appreciation for this help.
[b] Presented at the Nineteenth Annual Meeting of the Institute of Food Technologists, Philadelphia, May 18, 1959.



Figures 1, 3, 5, 7. Sections of peach tissue cooked in syrup which contained radioactive glucose. These are photomicrographs of unstained tissue embedded in parlodion.

Figures 2, 4, 6, 8. Corresponding autoradiographs (on nuclear track plates) of the sections shown in the odd-numbered figures to the left. Localization of radioactivity in the cell walls is especially to be noted. Figures 1–6, x 37. Figures 7, 8, x 15.

or subsequent microtechnical manipulation. In this way, unsupported sugar materials would lie against the cell wall and thus give that structure its relatively greater radioactivity. Although this may occur in part, the evidence suggests that these play a small role in the radioactivity accumulation in the cell wall. Firstly, the cell walls about large intercellular spaces have no more pronounced activity than other cell walls. Secondly, the cell walls of vascular elements, although surrounding relatively smaller lumina than parenchyma cell walls, have a much higher radioactivity than the latter. Moreover, although the vascular cell walls are considerably thicker than parenchymatous walls, they are still radioactive throughout. It is also to be noted that, the thicker the cell wall, the greater is its radioactive density. As a final point, it can be seen that radioactivity does exist in the cell lumen, indicating the presence of sugar particles there.

Consequently, it is apparent that radioactive glucose tends to accumulate in the cell walls in cooked peach tissue. Similar radioactively intense areas, particularly with respect to the vascular bundles, may be seen in nuclear track plates made from sections of canned cling-stone peaches *(3)*. Since most of these plates proved to be considerably under-exposed, they did not clearly reveal the cellular relationships described above.

That sugars may be adsorbed by cellulose, pectic substances, and other polysaccharides of the cell wall is not entirely unexpected. Even in cellulose, with most of the available hydroxyl groups well established in crystalline micelles *(1, 5)*, there are still some reactive groups available for hydrogen bonding. These exist at the surfaces of the micelles and along non-crystalline regions of molecules. The presence of such reactive groups explains the limited water sorption of cellulose *(2)*, its affinity for amylose *(4, 6, 7, 9)* and very likely for other hydroxyl-containing compounds, such as sugars. Less highly crystalline polysaccharides, such as pectic materials and hemicelluloses, would have even greater sorptive capacity than cellulose.

According to Kerr and Severson *(4)*, cotton will adsorb amylose to the extent of 1% of its own dry weight. The various shorter-chain derivatives of amylose (including glucose) would be expected to be similarly adsorbed on cellulose and other polysaccharides. In this light, the high concentration of sugar in the walls of vascular elements becomes comprehensible. These thickened walls have a larger number of polysaccharide molecules than their neighbors. Thus, they present a much greater surface of reactive hydroxyl groups to the solution. Thickened walls in epidermal and hypodermal cells and in sclereids should also adsorb significantly large amounts of sugar.

The concentration of sugar in the lumina of tracheary elements and in the intercellular spaces may be assumed to represent closely the concentration of sugar in the ambient solution. Since the former are as sparsely radioactive as the lumina of parenchyma cells, the cell walls have a greater concentration of radioactivity than does the ambient solution. Therefore, accumulation of sugar occurs against a concentration gradient. It is probable that adsorptive accumulation of sugar is an important factor in the diffusive movement of sugar into a canned fruit. It may be expected that sugar molecules will be absorbed in the cell wall until the tendency of a sugar molecule to be adsorbed equals its tendency to move off into the solution.

SUMMARY

Microscopic sections of peaches, which had been cooked (or canned) in syrups with radioactive sugars, were compared with autoradiographs made from these sections. Radioactivity was localized principally in the cell walls of the peach tissues. Thicker cell walls (in the vascular bundles) had a higher concentration of radioactivity than did thinner ones. These data indicate an adsorption of the sugars by the cell wall, probably against a concentration gradient.

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SOME EFFECTS OF SUGARS ON THE BREAKDOWN OF PELARGONIDIN-3-GLUCOSIDE IN MODEL SYSTEMS AT 90° C[a,b]

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(Manuscript received February 9, 1959)

It has been demonstrated that the three most important factors which determine the rate of breakdown of pelargonidin-3-glucoside, the major anthocyanin of strawberries, are pH, temperature and the presence of oxygen *(6, 9)*. Ascorbic acid has a marked effect and sugars also contribute to the breakdown of this pigment *(9)*.

Since sugars are present at such high concentration in many strawberry products and since there is a variety of sugars available for use in the preparation of these products, it was considered advantageous to study the effects of sugars on the rate of pigment breakdown. Meschter *(9)* has observed the rate of strawberry pigment degradation in the presence of different sugars at 38° C. Sugars such as arabinose, levulose and sorbose, which are relatively labile, produced a higher rate of pigment degradation than the more stable sugar maltose and the sugar alcohol sorbitol. The sugar degradation products, 5-hydroxymethylfurfural and furfural also increased the rate of degradation. Further work in model systems by Mackinney, Lukton and Chichester *(7)* showed fructose to be more active than sucrose. These workers also demonstrated an added effect in a sugar-amino acid system which was presumably due to the interaction of the sugar and amino acid. It has thus been suggested that sugars are active in the form of their degradation products in affecting the rate of pigment degradation and amino acids act by accelerating the production of these breakdown products. Decareau, Livingston and Fellers *(2)* have made some observations on the rate of loss of pigment in strawberry jellies. Contrary to expectation, replacement of sucrose by fructose increased the half life of the pigment.

The studies reported in this paper have been designed to investigate the effect of different sugars on the rate of degradation of pelargonidin-3-glucoside at 90° C incorporating such variables as pH, sugar concentration and amino acid interaction. A temperature of 90° C parallels processing rather than storage temperatures. An effort has been made to evaluate the relative importance of the factors associated with the degradation of the major pigment of strawberries.

EXPERIMENTAL PROCEDURE

Preparation of pelargonidin-3-glucoside. Pelargonidin-3-glucoside was obtained from strawberry juice using the procedure described by Sondheimer and Kertesz *(14)*. The

[a] Technical paper No. 1197, Oregon Agricultural Experiment Station. This research was financed in part by a grant from the Sugar Research Foundation, Inc.

[b] The data are from a thesis presented to the Graduate School of Oregon State College by Ian J. Tinsley in partial fulfillment of the requirements for the degree Doctor of Philosophy.

[c] Department of Agricultural Chemistry, Oregon State College.

crude product was converted to the picrate which was purified by recrystallization from hot dilute picric acid. The recrystallization was repeated 4 times. The anthocyanin chloride was precipitated with anhydrous ethyl ether from a solution of the picrate in 5% alcoholic hydrogen chloride. A final yield of approximately 700 mg of the purified pelargonidin-3-glucoside was obtained from 30 lb of strawberries.

Paper chromatograms of the pigment using a solvent system composed of equal volumes of *n*-butanol and 27% acetic acid showed only one spot, the Rf of which corresponded to that reported in the literature for pelargonidin-3-glucoside *(3)*. The spectrum of this preparation and that reported by Sondheimer and Kertesz *(14)* for pelargonidin-3-glucoside agree closely at all wave lengths from 220 to 570 mμc.

Preparation of 5-hydroxymethylfurfural. A small quantity of this compound was prepared by the method described by Haworth and Jones *(4)*.

Estimation of pelargonidin-3-glucoside. The method described by Sondheimer and Kertesz *(13)* was used for the estimation of pelargonidin-3-glucoside. Aliquots of the reaction mixture at pH 3.4 were withdrawn and added to an aliquot of citrate buffer which was adjusted so that dilution to the required concentration range resulted in a pH of 2.0 and 3.4 measured with a Beckman Zeromatic pH meter. On dilution of a sample to pH 2.0 it was necessary to allow at least an hour for the sample to come to equilibrium. Failure to do this resulted in considerable error in absorbance measurements at this pH. This effect was not observed in samples made up to pH 3.4. In systems where no interfering colored products were produced, the absorbance measurements at pH 3.4 were used to determine the pigment concentration. Absorbance measurements at 440 mμc indicated the production of interfering pigments such as those due to browning reactions.

Measurement of the pK of the equilibrium between the benzopyryllium salt and the pseudo-base form of pelargonidin-3-glucoside. The pK of this reaction at 86° C was determined using the method described by Sondheimer *(12)*. Pelargonidin-3-glucoside was dissolved in solutions of hydrochloric acid, the pH of which varied from pH 1.0 to 3.0. The absorbance of these solutions was measured at room temperature and at 86° C. The measurements at 86° C were obtained by using a "Thermospacer" attachment with the Beckman Model DU spectrophotometer. The pK of this reaction was then calculated from these measurements as described in the above mentioned paper.

Rate studies. Three 100 ml three-necked standard taper round-bottom flasks, fitted with air inlet tubes, condensers and thermometers, were mounted in a constant temperature bath. The rate of air flow was controlled for each individual flask at 160–170 cc/min measured at standard temperature and pressure. In this manner the reaction mixture was saturated with air and kept well stirred. The temperature of the heating bath was adjusted to give a temperature of 90.0 ± 0.2° C in the reaction flask.

In carrying out a rate study a 40 ml solution containing all the required components except the citrate buffer and pigment was allowed to come up to temperature (approximately 10 min). At this time 5 ml of citrate buffer and 5 ml of distilled water containing 10 mg of pigment were added. The buffer was prepared in such a manner that a 1:10 dilution resulted in 0.1*M* citrate buffer of pH 3.4. The final volume of solution was 50 ml and the reaction mixture was 0.427 m*M* with respect to the pigment. As soon as the temperature of the reaction mixture came up to 90° C (2–3 min) an aliquot was withdrawn and the pigment concentration of this aliquot was taken as that at zero time. At definite time intervals aliquots were withdrawn and the concentration of pelargonidin-3-glucoside determined. When reaction rates were measured in the presence of nitrogen, water pumped nitrogen was used and the solutions were flushed with nitrogen before the rate studies were commenced. In systems containing ascorbic acid or amino acids these components were added along with the buffer and pigment. The reaction was followed until the original pigment concentration had been reduced to at least one half; in all cases a minimum of 7 readings was taken.

RESULTS

Pigment degradation in the presence of sugars and their derivatives. The degradation of pelargonidin-3-glucoside was observed in 0.5*M* solutions of the monosaccharides and 0.25*M* solution of the disaccharides. These concentrations approximate the soluble

solids content in strawberry juice. A pH of 3.4 was maintained with 0.1*M* citrate buffer and the reactions were followed in the presence of air and nitrogen.

Graphical analysis of the results from the rate studies indicated that in most cases the rate of disappearance of pelargonidin-3-glucoside followed first order kinetics. The rate of pigment degradation in different systems can thus be expressed by the first order rate constant. In sucrose systems first order kinetics were not attained until the reaction had proceeded for approximately 120 min. First order rate constants quoted for sucrose systems refer to the portion of the rate curve where t is greater than 120 minutes.

In Table 1 the sugars used and the rate constants, both in air and nitrogen, are listed. The percent reduction in rate constant observed in the presence of nitrogen is also tabulated. In the presence of air the rate of pigment degradation in 0.25*M* sucrose is approximately half that observed in the 0.5*M* fructose system. This would suggest that the lag observed is due to the hydrolysis of the sucrose.

These results show that sugars such as fructose and glucuronic acid, which are known to degrade quite rapidly at low pH, have a pronounced effect on the rate of pigment degradation. Glucuronic acid decomposes to furfural and fructose to 5-hydroxymethylfurfural. Sorbitol and gluconic acid, which are stable under the conditions of these experiments, show degradation rates comparable to that observed in the buffer system.

In the presence of nitrogen marked reductions in the rate of pigment degradation are observed. However, differences in the rate of pigment degradation are still observed with the sugars used. The relative reduction observed in the rate constant was not the same for all sugars used. The rate of pigment breakdown in the gluconic acid and sorbitol systems was virtually the same in air as it was in nitrogen.

Effect of sugar-amino acid interaction on the rate of degradation of pelargonidin-3-glucoside. Experiments conducted by Mackinney, Lukton and Chichester *(7)* indicated that pelargonidin-3-glucoside was decomposed more rapidly in a sugar solution containing 1% glycine than in a pure sugar or pure glycine solution. A 1% glycine solution would be somewhat more concentrated than one would expect in strawberry juice. In the studies carried out in this laboratory the effect of sugar-amino acid interaction on the rate of pigment degradation has been observed at amino acid concentrations of the same order as that found in strawberry juice *(16)*. For each amino acid used the rate of pigment degradation was observed in a pure amino acid system, amino acid plus 0.5*M* glucose and amino acid plus 0.25*M* sucrose. In each case except cystine the amino acid concentration was $2.5 \times 10^{-3}M$ and all systems were buffered to pH 3.4 with 0.1*M* citrate. The amino acids used were representative of the different functional types found in strawberries *(16)*.

In these systems also, the rate of decomposition of pelargonidin-3-glucoside followed first order kinetics. The rate curve for sucrose systems was similar to that observed previously in these studies for sucrose alone. In Table 2 the first order rate constants are tabulated for the different systems studied.

TABLE 1

First order rate constants for the degradation of pelargonidin-3-glucoside in different carbohydrate systems

Carbohydrate	First order rate constants ($min^{-1} \times 10^2$)		Percent reduction
	Air	Nitrogen	
Glucose	2.60	1.93	26.0
Fructose	7.91	2.80	65.0
Sucrose	3.96	2.88	27.0
Sorbitol	2.04	2.16	0.0
Gluconic Acid	2.14	2.15	0.0
Glucuronic Acid	14.70	10.00	32.0
Maltose	2.66	2.07	22.0
Lactose	3.50	2.32	34.0
Buffer	2.02	1.77	12.5

TABLE 2

First order rate constants for the degradation of pelargonidin-3-glucoside in the presence of sugar-amino acid systems

Amino acid	First order rate constants ($min^{-1} \times 10^3$)		
	Buffer	Glucose	Sucrose
Asparagine	1.96	3.09	4.24
Alanine	2.09	2.86	4.49
Serine	2.05	2.92	4.54
Arginine	2.36	3.04	4.90
Cystine*	2.53	3.58	4.96
No Amino Acid	2.02	2.60	3.96

* Concentration 1.25×10^{-3}M.

In some preliminary experiments the amino acids were heated in a 0.5*M* glucose solution buffered at pH 3.4 for 12 hr. The ultra-violet spectra of these systems showed a maximum absorbancy at 285 mmc. This maximum is characteristic of 5-hydroxymethylfurfural. Arginine and cystine were the most active acids in these systems. It would appear that the extent to which amino acid-sugar interaction affects the rate of pigment degradation is dependent on the extent of sugar degradation as measured by the appearance of 5-hydroxymethylfurfural.

Effect of variation in sugar concentration on the rate of degradation of pelargonidin-3-glucoside. The rate of degradation of pelargonidin-3-glucoside was observed in varying concentrations of glucose, sucrose and fructose at pH 3.4. In all cases an increase in the sugar concentration resulted in an increase in the rate of pigment degradation at pH 3.4 and 90° C.

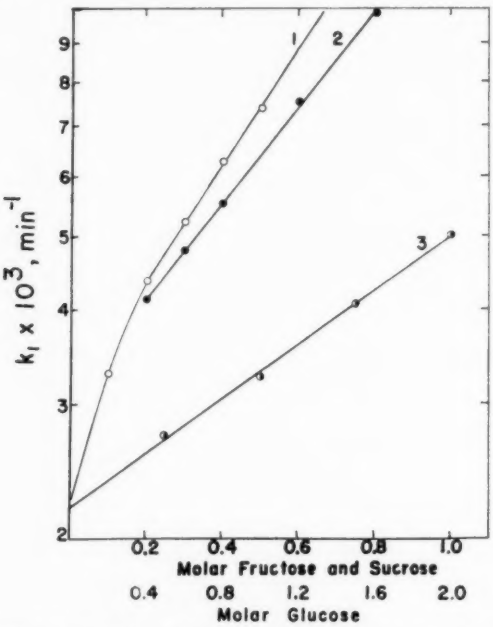


Figure 1. Effect of variation in sugar concentration on the rate of degradation of pelargonidin-3-glucoside. (1) fructose, (2) sucrose, (3) glucose.

In Figure 1 the logarithm of the first order rate constant is plotted against the sugar concentration. Over most of the concentration range investigated, a linear relation was observed. The reason for this relation is not apparent.

The effect of pH on the rate of degradation of pelargonidin-3-glucoside in sugar solutions. It has been shown that pelargonidin-3-glucoside is more stable at lower pH *(6)*, but on the other hand it is known that the rate of degradation of both glucose and fructose to 5-hydroxymethylfurfural increases as the pH decreases *(11)*. It has also been demonstrated *(8, 9)* that 5-hydroxymethylfurfural is active in accelerating the degradation of strawberry pigment. Thus, two apparently counteracting variables are in existence in this system. In some preliminary experiments ultra-violet spectra of glucose and fructose solutions heated at pH 3.4 indicate the presence of 5-hydroxymethylfurfural. The rate of degradation of pelargonidin-3-glucoside was followed in 0.1*M* citrate buffer and in 0.5*M* glucose and 0.1*M* fructose solutions also buffered with 0.1*M* citrate. The pH of these systems was varied from 3.95 to 1.80 and the reactions were carried out in the presence of air.

In order to interpret the results it was necessary to obtain an estimate of the pK of the reaction $R^+ + 2H_2O = ROH + H_3O^+$ where R^+ represents the colored flavylium salt form of the anthocyanin and ROH the pseudo-base form. A value of 2.98 for the pK of this reaction at 25° C and 45° C has been reported *(6, 12)*. No values for higher temperatures have been reported. Using the procedure outlined in the Experimental section, a value of 2.95 at a temperature of 86° C was obtained. This value is within the range of 2.98 ± 0.06 reported by Sondheimer *(12)*. Hence, in interpreting these results from experiments carried out at 90° C it would seem that a pK of 2.98 would be a valid approximation.

The citrate buffer maintained the pH of the systems within 0.05 of a pH unit. The rate of pigment loss followed first order kinetics in all the systems studied in

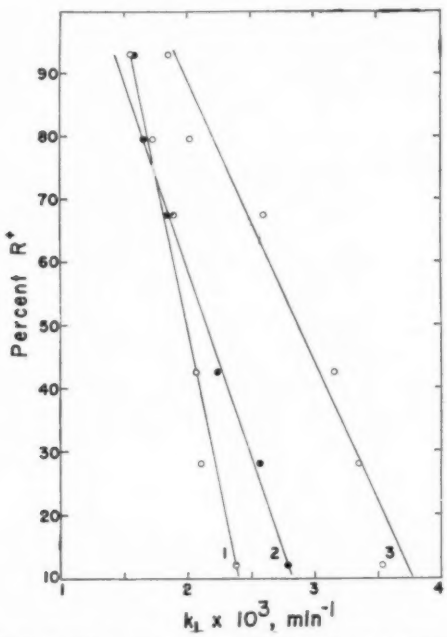


Figure 2. Effect of pH on the rate of degradation of pelargonidin-3-glucoside in 0.1M citrate buffer, 0.5M glucose and 0.1M fructose. (1) citrate buffer, (2) glucose, (3) fructose.

this series of experiments. Results are presented in Figure 2 where the rate constants are plotted against the percentage of the anthocyanin present as the flavylium salt form. The linear relation observed in the 3 systems studied implies that, over the pH range investigated, the degradation of the anthocyanin is dependent on the proportion of anthocyanin present as the pseudo-base. Lukton, Chichester and Mackinney *(6)* observed this effect in buffer systems, as well as in juice samples at a temperature of 40° C. Results from the fructose system are not as consistent as in the other 2 systems but the general trend is the same. As the pH decreases the differences in rate constants for the 3 systems also decrease.

The effect of some sugar degradation products on the rate of degradation of pelargonidin-3-glucoside. 1. *Furfural and 5-hydroxymethylfurfural.* The increase in pigment degradation in the presence of furfural and 5-hydroxymethylfurfural has been described by Meschter *(9)* and Markakis, Livingston and Fellers *(8)*. These studies were carried out at 38° C and 40° C respectively. Meschter's results indicate that, at equivalent concentrations, furfural is considerably more active than 5-hydroxymethylfurfural at 38° C. The concentrations, however, are quoted in parts per million and a rough interpretation of these results on the basis of equivalent molar concentrations indicates only slight differences in activity between these 2 compounds at this temperature.

The effect of furfural and 5-hydroxymethylfurfural on the rate of degradation of pelargonidin-3-glucoside at 90° C and pH 3.4 was followed. The reactions were carried out both in the presence of air and in the presence of nitrogen. The furfural was distilled under a vacuum in an atmosphere of nitrogen before use. The first order rate constants obtained are listed in Table 3. Degradation rates were also observed in 0.1*M* furfural but first order kinetics were not observed in either air or nitrogen. A considerable reduction in the rate of pigment degradation was observed when the reaction was carried out in the presence of nitrogen rather than air. The reaction rate, however, was not reduced to that observed in a buffer system in the presence of nitrogen. Either there was a residual effect from a small amount of oxygen that might have been present or these compounds do have some effect in the absence of oxygen.

TABLE 3

First order rate constants for the degradation of pelargonidin-3-glucoside in the presence of furfural and 5-hydroxymethylfurfural at pH 3.4

Concentration	First order rate constant ($min^{-1} \times 10^3$)			
	Furfural		5-Hydroxymethylfurfural	
	Air	Nitrogen	Air	Nitrogen
0.01*M*	2.88	1.87	.....	.....
0.05*M*	4.66	2.44	17.8	7.29

The rate of pigment degradation was also observed in 0.05*M* solutions of furfural and 5-hydroxymethylfurfural at different pH values. As in previous experiments 0.1*M* citrate buffer was used. First order kinetics were observed and the rate constants are plotted against the percentage of the anthocyanin present as the flavylium salt (Figure 3). Values obtained at the lower pH region suggest a linear relation but the rate constants observed at pH 3.4 (Table 3) were not consistent with this relation.

Both furfural and 5-hydroxymethylfurfural produced a marked effect on the rate of destruction of pelargonidin-3-glucoside. The magnitude of this effect was considerably reduced by carrying out the reaction in the presence of nitrogen or reducing the pH. 5-Hydroxymethylfurfural was consistently more reactive than furfural at equimolar concentrations. These experiments, carried out in systems of varying pH, would suggest that the pseudo-base form of the anthocyanin was the active form in these systems also. It cannot be concluded from these studies that the pigment reacts directly with either of these 2 compounds. Both are very labile, particularly in the presence of oxygen and the degradation of the pigment could possibly be due to the presence of some oxidative product derived from these aldehydes. However,

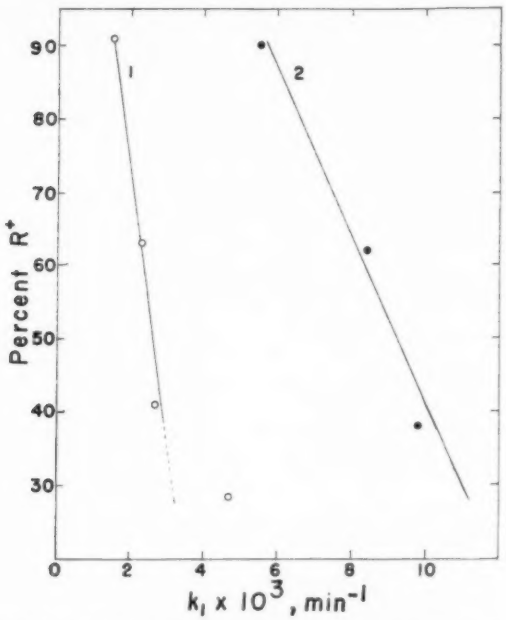


Figure 3. Effect of pH on the rate of degradation of pelargonidin-3-glucoside in the presence of furfural and 5-hydroxymethylfurfural. (1) furfural, (2) 5-hydroxymethylfurfural.

in a system in which either of these 2 compounds was produced in some fashion, one could expect a significant rate of pigment degradation. Thus, the degradation of sugars in the presence of acid or amino acids, which result in the production of these types of compounds, produces an increase in the rate of pigment breakdown. It is possible that the effect of ascorbic acid could be due in part to its breakdown to furfural.

2. *Levulinic Acid and Formic Acid.* In acid medium 5-hydroxymethylfurfural is degraded to levulinic acid and formic acid. The kinetics of this reaction have been studied by Teunissen *(15)*. Because of the possibility that either formic or levulinic acid was the reactive compound in pigment breakdown, the effect of levulinic acid and formic acid on the breakdown of pelargonidin-3-glucoside was observed. The reactions were carried out in 0.1*M* citrate buffer at pH 3.4 and at a temperature of 90° C. The first order rate constants obtained are given in Table 4.

The rate of pigment degradation in these systems, although greater than that observed in a buffer system of the same pH, was much slower than that observed in

TABLE 4

First order rate constants for the degradation of pelargonidin-3-glucoside in the presence of levulinic acid and formic acid

Concentration	First order rate constant ($min^{-1} \times 10^3$)	
	Formic acid	Levulinic acid
0.10*M*	3.15	3.68
0.05*M*	2.55	2.75
0.01*M*	2.25	2.40

equimolar concentrations of 5-hydroxymethylfurfural. Thus, it is apparent that the accelerating effect of 5-hydroxymethylfurfural is not dependent on its breakdown to levulinic acid and formic acid. It might be added that in the breakdown of 5-hydroxymethylfurfural other substances besides these 2 acids are formed. A certain amount of resinous material is produced.

The relative effect of factors active in the degradation of pelargonidin-3-glucoside. In strawberry juice the constituents which appear to be most active in determining the rate of pigment degradation are ascorbic acid, sugars and amino acids. A sample of strawberry juice obtained from frozen Marshall strawberries was analyzed for pigment concentration, ascorbic acid, soluble solids and pH. Results are summarized in Table 5.

TABLE 5

Analysis of strawberry juice sample

Soluble Solids	10.0%
Ascorbic Acid	20 mg/100 ml
Pigment	22 mg/100 ml
pH	3.35

The rate of degradation of pelargonidin-3-glucoside in a sample of this juice adjusted to pH 3.4 with citric acid was compared with the rate of degradation of this pigment in the following systems:

1. 0.5*M* glucose
2. 0.5*M* glucose plus amino acids
3. 0.5*M* glucose plus ascorbic acid
4. 0.5*M* glucose plus ascorbic acid plus amino acids

These experiments were repeated with 0.25*M* sucrose substituted for the glucose. The concentration of ascorbic acid was 20 mg per 100 ml (Table 5) and the amino acids consisted of a mixture, of the five most abundant acids found free in strawberry juice, present in the following concentrations:

Asparagine	59 mg/100 ml
Glutamine	15 mg/100 ml
Alanine	12 mg/100 ml
Aspartic Acid	3 mg/100 ml
Glutamic Acid	8 mg/100 ml

The rate curves for the sucrose systems are given in Figure 4; for comparison the rate curve of pelargonidin-3-glucoside degradation in citrate buffer is also included. Both glucose and sucrose systems give a more rapid rate of pigment degradation than that observed in a buffer system, sucrose being the more active during a three-hour period. Adding the amino acids to the sugars resulted in an additional increase in the rate of pigment degradation. In the sugar plus ascorbic acid system there was little difference between the glucose and sucrose systems, a marked increase in the rate of pigment breakdown being observed on addition of ascorbic acid. Further addition of the amino acids to the sugar-ascorbic acid produced a small increase in the rate of pigment degradation. It was noted that in the latter system, after an initial lag period which was not observed in the juice sample, the rate of pigment degradation approaches that observed in strawberry juice. This would indicate that these 3 factors account for the greater part of the pigment breakdown in strawberry juice.

The juice sample showed first order kinetics for the degradation of the pigment. The fact that no lag was observed in the juice sample may be explained by the assumption that the active principles were already present; with the synthetic sample a short time was necessary before these factors were produced at a rate sufficient to equal the rate of pigment breakdown in the juice. The ultra-violet spectrum of an ether extract of strawberry juice showed a maximum at 283 mmc. This corresponds to the maximum absorption of 5-hydroxymethylfurfural.

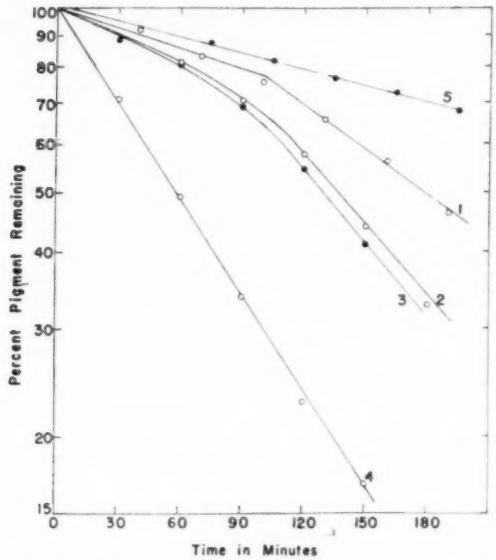


Figure 4. Comparison of the rate of degradation of pelargonidin-3-glucoside in composite systems with that observed in a strawberry juice sample. 1. Sucrose and amino acids. 2. Sucrose and ascorbic acid. 3. Sucrose, amino acids and ascorbic acid. 4. Strawberry juice. 5. Citrate buffer control.

DISCUSSION

The stability of pelargonidin-3-glucoside varied considerably in the different systems studied in these experiments. A hundredfold difference in first order rate constant was observed at 90° C. The rate of degradation of pelargonidin-3-glucoside in most of the systems studied followed first order kinetics, as reported by Lukton, Chichester and Mackinney *(6)* for citrate buffered model systems and in strawberry juice in the presence or absence of oxygen. Markakis, Livingston and Fellers *(8)*, however, working with citrate buffer systems also, observed first order kinetics in the presence of nitrogen but not in the presence of oxygen. It is possible that in the latter experiments the oxygen concentration became limiting since these data were obtained from sealed tube experiments. In the systems used by Lukton *et al (6)* and in this laboratory the reaction mixture was saturated with oxygen by continually agitating with air or oxygen.

Perhaps the most striking effect in the work reported here was the increase in pigment stability associated with a decrease in pH. This increase in stability was observed even in the presence of active components such as furfural and 5-hydroxymethylfurfural. In the presence of glucose or fructose, as well as in a buffer system, a linear relation was observed between the first order rate constant and the relative concentration of the anthocyanin present as the pseudo-base. Lukton *et al*, working with citrate buffer systems and natural strawberry juice, have observed this type of relation

and suggest that the active form of the pigment is the pseudo-base form. Results obtained in this laboratory substantiate this hypothesis.

The important part that sugars can play in the degradation of pelargonidin-3-glucoside is demonstrated by the detrimental effect of fructose and glucuronic acid in particular. Strawberry products may contain considerable quantities of fructose derived from the hydrolysis of sucrose. Although glucuronic acid would not be present in strawberry juice in significant amounts, galacturonic acid could be obtained from the hydrolysis of pectin material. Since galacturonic acid, like glucuronic acid, degrades rapidly to furfural in acid medium, one would obtain a comparable effect on the rate of pigment degradation. Fructose would be of more importance since it is more likely that it would be present in concentrations sufficient to produce a significant effect.

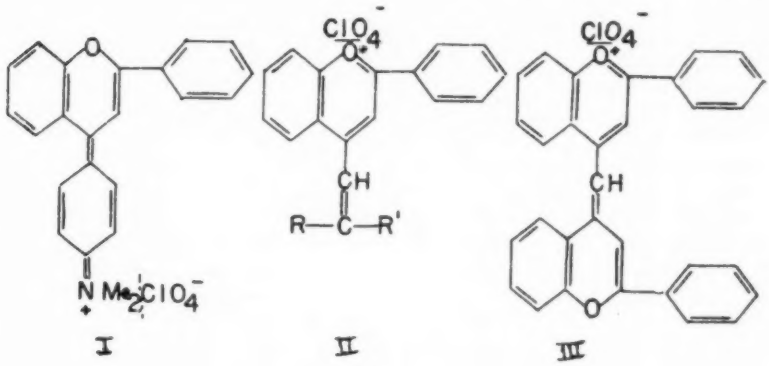
At 90° C none of the sugars used in these experiments exerted a protective effect on the pigment. At lower temperatures the pigment has been shown to be more stable in some sugar solutions (longer half life) than in a buffer system *(9)*. This could be due to differences in oxygen concentration in the different systems where sugar degradation products were not a factor.

Amino acids also have an effect on the rate of pigment degradation in the presence of sugars. The results indicate that arginine and cystine may have some effect on the rate of degradation of pelargonidin-3-glucoside in a buffer system. Even at pH 3.4 where amino acid-sugar interaction is relatively low, the presence of amino acids in the glucose and sucrose systems produced an increase in the rate of pigment degradation. There is some question in the arginine-glucose system whether the increase is due to the arginine or arginine-glucose interaction. The increase in rate constant over that observed in a pure glucose system is only slightly larger than the increase in rate constant of the arginine-buffer system above that of a pure buffer system. Cystine proved to be the most active of the amino acids tested. The data obtained in these studies indicate, however, that the amino acids present in strawberry juice do not contribute to the rate of degradation of the pigment to any large extent. The low pH of strawberry juice would tend to minimize the interaction of sugars and amino acids.

The experimental data indicate that the sugars are probably active in the form of their degradation products. Those sugars such as fructose which degrade most readily in acid medium show the most marked effect on the rate of pigment degradation. An increase in sugar concentration also results in an increase in the rate of pigment degradation. The compounds furfural and 5-hydroxymethylfurfural, which have been identified as sugar breakdown products *(10)*, accelerate the degradation of pelargonidin-3-glucoside. The rate of degradation of pelargonidin-3-glucoside in the presence of these compounds, as in the presence of sugars, is decreased by lowering the pH and also by conducting the reaction in the presence of nitrogen rather than air. The marked increase in the rate of pigment degradation in the presence of these 2 compounds suggests that they might be directly responsible for degradation of the pigment in the presence of sugars. Since both furfural and 5-hydroxymethylfurfural are quite labile in the presence of oxygen, possibly some oxidative product might be the

reactive compound, although typical breakdown products such as levulinic and formic acids were not effective in degrading the pigment. Another possibility is that in the presence of sugars some precursor of either furfural or 5-hydroxymethylfurfural could be the active component. At present the exact nature of the degradation of pelargonidin-3-glucoside in the presence of sugars is not known. More information is needed, both on the nature of the degradation of sugars in acid medium and the interaction of these products with anthocyanins.

Recent publications on the reactions of flavylium salts by Wizinger and Luthiger *(17)* and Blackburn, Sankey and Alexander *(1)* indicate a possible mode of action for the degradation of pelargonidin-3-glucoside in the presence of active components such as furfural and 5-hydroxymethylfurfural. In these papers the condensation of flavylium salts with dimethylaniline diarylethylenes and malonic acid to form the products I, II, and III has been described. The significant aspect of these reactions is that the antho-



cyanin is active in the form of the pseudo-base and also the reaction does not take place in the absence of oxygen. The reaction is described as an "oxidative coupling." There is a marked similarity to the conditions required for the degradation of pelargonidin-3-glucoside, where the anthocyanin reacts in the form of the pseudo-base and oxygen has a pronounced effect on the rate of degradation.

Investigations on the effects of substituents on the benzopyran ring indicate that although a reaction was observed the rate was much slower with 3- and 5-substituted flavylium salts. Since pelargonidin-3-glucoside has substituents in these two positions, it might be argued that this would preclude this type of reaction as an explanation of pigment degradation. However, the extent of the reaction would also be a function of the reactivity and size of the attacking group, as well as the nature and position of the substituents of the benzopyran ring.

Ascorbic acid is probably the most important constituent in strawberries relative to pigment degradation. The exact nature of the action of ascorbic acid is not known. It has been suggested that it may be due to hydrogen peroxide formed on oxidation of the ascorbic acid, but this mechanism has

not been established. Since furfural has been demonstrated to be a degradation product of ascorbic acid in acid medium *(5)*, acorbic acid, in a similar manner to sugars, may owe its activity to its conversion into this type of compound.

SUMMARY AND CONCLUSIONS

1. In systems buffered at pH 3.4 the rate of degradation of pelargonidin-3-glucoside was observed in the presence of different sugars and sugar derivatives. Variation of the functional group indicated that the rate of pigment degradation was greatest in the presence of glucuronic acid and the ketose, fructose. Sorbitol, a sugar alcohol and gluconic acid gave the slowest rates of pigment degradation. The rate of pigment breakdown in the presence of sugars seemed to be associated with the rate at which the sugar degenerated to furfural type compounds. If the reactions were carried out in the presence of nitrogen rather than air a marked decrease in the rate of pigment degradation was observed.

2. The presence of amino acids, at concentrations of the same order as those observed in strawberry juice, in sucrose and glucose systems produced an increase in the rate of pigment degradation compared to that observed in the presence of a pure sugar or pure amino acid system.

3. At 90° C and pH 3.4 it was found that an increase in the concentration of glucose, fructose or sucrose produced an increase in the rate of pigment degradation. A plot of the logarithm of the observed rate constants versus the sugar concentration resulted in a linear relation over the concentration range observed.

4. The rate of degradation of pelargonidin-3-glucoside was influenced to a great extent by pH. This effect may be explained by the influence of hydrogen ion concentration on the equilibrium between the pseudo-base and the flavylium salt form of the anthocyanin. Linear relations were observed between the first order rate constants and the proportion of the pigment present as the flavylium salt in glucose and fructose systems as well as the pure buffer system. The rate of degradation seemed to be dependent on the proportion of the anthocyanin present as the pseudo-base.

5. A rapid rate of degradation of pelargonidin-3-glucoside was observed in the presence of furfural and 5-hydroxymethylfurfural. Carrying out the reactions in nitrogen rather than air resulted in a considerable reduction in the rate of pigment degradation. Observations on the rate of pigment degradation in the presence of furfural and 5-hydroxymethylfurfural in systems of varying pH also indicated that the active form of the pigment was the pseudo-base form.

6. Levulinic acid and formic acid, degradation products of 5-hydroxymethylfurfural in acid medium, accelerate the rate of pigment degradation. However, the effect observed with these compounds was small compared to that observed with 5-hydroxymethylfurfural. This would indicate that the accelerating effect of 5-hydroxymethylfurfural was not dependent on its breakdown to these compounds.

7. A composite system containing pigment, sucrose or glucose, amino acids and ascorbic acid at concentrations similar to those observed in a sample of strawberry juice, after an initial lag period, gave a rate of pigment

degradation almost equal to that observed in the natural strawberry juice sample. These factors would apparently account for the greater part of the pigment degradation in strawberry products.

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OXIDATION OF FAT IN MODEL SYSTEMS RELATED TO DEHYDRATED FOODS[a]

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Recent development of a great variety of dehydrated quick-serve ration items by the Quartermaster Food and Container Institute for the Armed Forces has again emphasized the need for means of inhibiting oxidative deterioration of the dispersed fats. In planning the present study, a model system approach was decided upon as the method of choice since it permitted investigating oxidation problems in their most general manifestations. This approach also provided for closer control of the variables than would be possible with food items. Model emulsion systems designed to simulate dehydrated food hydrocolloids were therefore prepared and studied under accelerated storage conditions for deteriorative reactions.

Fresh foods are known to contain natural substances which protect them from rapid deterioration. For example, antioxygenic compounds, among which are tocopherols and phospholipids, have an important function in minimizing the deterioration of food fats. There are, of course, substances that promote deterioration. Heme compounds are widely distributed in foods of animal origin, and their importance in the oxidative fat rancidity which occurs in these foods is well recognized. In these studies hemoglobin and hematin were used as oxidative catalysts.

The antioxygenic properties of vegetable phospholipids have been studied by Evans *(3)*, Olcott and Mattill *(8)*, and Patrick and Morgan *(9)*; the antioxygenic action of egg phospholipids has been demonstrated by Dutton and Edwards *(2)*, Lightbody and Fevold *(7)* and more recently by Henick, Togashi and Mitchell *(4)*. Brown and Tappel *(1)*, Robinson *et al (10)*, Tappel *(14, 15)*, and Watts *(16)* have shown that hemoglobin and other heme compounds act as powerful biological catalysts for oxidation of fat.

The purpose of these studies was to determine by means of model dehydrated colloid systems what effect phospholipids and proteins have on rates of oxidation of dispersed fat in systems which may contain oxidation-promoting hematin compounds. The study was, of necessity, exploratory in nature. Rates of reaction of fat and other food components in the newly developed freeze-dried foods have not been studied extensively, and as a consequence background data are limited. The simplified model system approach to the problem offers the possibility of developing information applicable to the control of oxidative deterioration in dehydrated foods containing dispersed fat.

EXPERIMENTAL

Methods. Dehydrated fatty emulsions with an inert matrix of carboxymethyl cellulose (CMC) were used to evaluate the roles of proteins and phospholipids as antioxidants

[a] This paper reports research conducted by the Quartermaster Food and Container Institute for the Armed Forces and has been assigned No. 1004 in the series of papers approved for publication.

and of iron compounds as pro-oxidants in accelerated lipid oxidation experiments. Dehydration was carried out by means of freeze-drying similar to that described by Rolfes *et al (11)*. Samples were aged by aerating them in a rancimeter *(6)* at elevated temperatures to permit acceleration of oxidative reactions. The method used was similar to that described by Salwin *et al (12)*. Extent of oxidation was evaluated by means of the ferric thiocyanate method of Hills and Thiel *(5)* for assaying peroxides spectrophotometrically at 510 mmc. Carbonyl and other compounds, which were collected as volatiles from aerated samples, were assayed by means of 2-thiobarbituric acid (TBA) reagent using a method similar to that described by Sidwell *et al (13)*.

Materials

1. Carboxymethyl cellulose gum (CMC) (Hercules Powder Co.).
2. Refined edible soy oil.
3. Soy phospholipids—composed approximately of one-third lecithin, one-third cephalin and one-third inositides.
4. Soy proteins (J. R. Short Milling Co.), food grade, ground to a very fine powder in a ball mill.
5. Hemoglobin (purified) (Mann Research Co.).
6. Hematin, recrystallized (Mann Research Co.).
7. Iron citrate (ferric) 16–18% Fe (Merck & Co.).

Preparation of dehydrated emulsions. All emulsions were prepared with CMC dispersed in water. The various food components under study were added in the following sequence: (1) iron compounds, (2) phospholipids, (3) oil, and (4) proteins. One minute was allowed for blending each component in a Waring blender. Total solids in the emulsion were adjusted to 15% by the addition of water. Emulsions were then immediately shell-frozen in round-bottomed flasks and dehydrated by freeze-drying. Dried emulsions were ground in a sharp-bladed Waring blender. This resulted in very light and fluffy flakes of a very large surface area resembling foam rubber and having a density of 0.1–0.2 g/cc.

RESULTS AND DISCUSSION

Effects of emulsification and dehydration. A series of experiments was conducted to determine the effects of dispersion on the rates of autoxidation of fats in dehydrated simplified emulsions. The data (Table 1) indicate that the physical dispersion of soy oil significantly accelerates its rate of autoxidation as compared to the same oil in the fluid state. As indicated in Table 1, there was no oxidation until the seventh day of storage. Oxidation continued at a slow rate for the next 10 days. The rate of oxidation of the

TABLE 1

Effect of added phospholipids on the autoxidation of oil in freeze dehydrated carboxymethyl cellulose (CMC) emulsion

Storage at 50° C	Peroxides of oils[1]		
Days	Fluid	Emulsified-dehydrated	Phospholipids added dehydrated
0	None	3.2	0.6
1	None	14.7	1.2
3	None	29.4	2.1
5	None	48.5	2.6
7	0.3	37.1	3.1
12	1.2	30.3	2.0
17	2.2	39.9	3.1

[1] Absorbancy per gram of oil in accordance with the Hill and Thiel method.

dispersed oil in the dehydrated emulsion was much more rapid, reaching a maximum after 5 days, followed by partial peroxide breakdown until the end of the storage period. Adding phospholipids prevented the autoxidation of the dispersed oils to a significant extent. The dispersed oil emulsion without phospholipid treatment was definitely rancid after 17 days, whereas the dehydrated oil emulsion with phospholipids was free of rancid odor and compared well with the fluid oil under study.

The protective effect of phospholipid in dehydrated emulsions containing CMC, soy oil, and protein was studied at different temperatures and different levels of soy phospholipids (Figure 1). As would be expected, increasing amounts of phospholipids were required to inhibit fat oxidation as temperature was increased. The apparent reversal in peroxide production at 65° and 80° C was undoubtedly due to a faster rate breakdown of peroxides at the higher temperature. Also of interest was the observed protection of phospholipid at −20° C. It was noted that rancid odor typical of oxidized fat did not occur in the presence of phospholipid even at the higher peroxide levels or when peroxide breakdown was presumed to occur. Similar effects were observed when purified egg phospholipids and soy inositides were used.

Catalytic effect of iron compounds on fat oxidation. The activity of iron containing pro-oxidants was studied on a comparative basis in dehydrated and fluid systems. In Figure 2 the rehydrated emulsions are indicated by solid curves. These emulsions consisted of two parts soy oil and one part

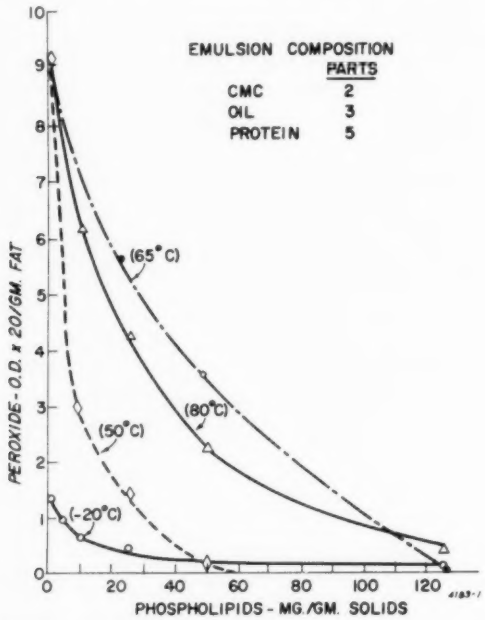

Figure 1. Relation of phospholipid levels to rates of autoxidation at various temperatures for 5 days.

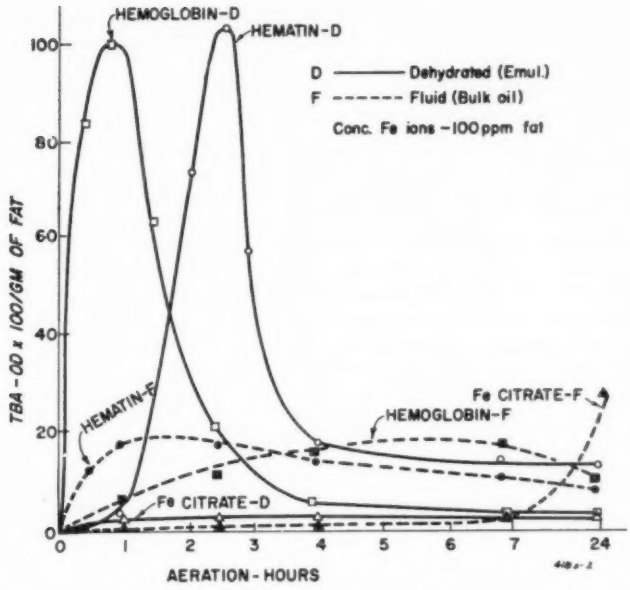

Figure 2. The effect of iron compounds in soya bean oil on rate of oxidation.

CMC with 100 ppm (oil basis) of the iron in the compound added. In the fluid oils these iron compounds were dispersed by means of a superhomogenizer. TBA assays for the dehydrated emulsions were made on the collected volatiles from the rancimeter apparatus. Volatiles from the fluid oil emulsions were collected from the effluent stream of standard AOM tubes, using the AOM apparatus. Collection of volatiles was carried out during the last 15 minutes of each interval shown. Examination of these data indicated that the rates of oxidation, as assayed by TBA values, were approximately five-fold more rapid for oils in dehydrated emulsions than in the fluid systems. Most of the differences observed are due to the high degree of dispersion of the oil in the dehydrated emulsions. The effects of the degree of dispersion of the oil

TABLE 2

Spontaneous combustion in freeze dehydrated vegetable oil-CMC emulsions in a draft oven at 90° C

Additives[1]	Time (hours) to reach spontaneous combustion[2]
Hemoglobin	0.2
Hematin	1.5
Hemoglobin + phospholipids	4.0
Ferric citrate	8.0
None	10.0
Phospholipids	24.0

[1] All emulsions contained oil dispersed in CMC with the additive as indicated above.
[2] Spontaneous combustion was indicated by charring. The temperature at the site of combustion (smouldering) was between 150° C–175° C.

in these emulsions on oxidation rates, when freeze dehydrated, will be reported in separate communications. The extremely rapid oxidation of hemoglobin containing dehydrated samples resulted in these emulsions becoming overheated, smouldering, and approaching spontaneous combusion. At shown in Figure 2, the rate of production of TBA reactive compounds for hemoglobin containing emulsion reached a maximum in less than 1 hour; for those with hematin it took more than 2 hours; and for those with ferric citrate, even after 24 hours, no significant oxidative action was indicated. Actual destruction of hemoglobin containing samples by spontaneous combustion occurred in samples heated in a draft oven at the temperature indicated in data in Table 2. Comparison of oxidation rates here indicates that hemoglobin acts as a very powerful pro-oxidant while the phospholipids are effective antioxidants when added to these dehydrated emulsions.

Effect of hemoglobin on oxidation rate. Of the several iron compounds investigated, hemoglobin was found to be the most powerful autoxidative catalyst. Concentrations ranging from 5–100 ppm as Fe were incorporated in

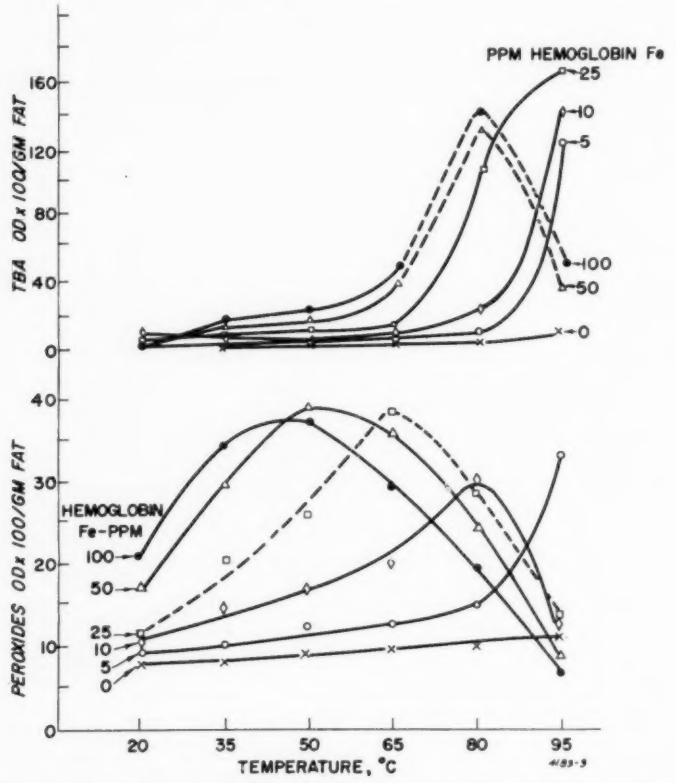

Figure 3. The effects of hemoglobin and temperature on the rate of oxidation in dehydrated emulsions. (Aeration time—one hour).

dehydrated emulsions containing 2 parts oil in 1 part CMC. The data (Figure 3) indicate that rates of autoxidation of the oil during one hour increased with increasing concentrations of hemoglobin. The temperature at which the rate of peroxide decomposition exceeded the rate of formation decreased with increasing hemoglobin concentration. The formation of TBA reactive volatiles appears related to the decomposition of peroxides.

Effect of proteins and phospholipids on the rate of oxidation. A study of the effect of the addition of protein to a CMC-oil mixture, which was emulsified and dehydrated, showed that the protein had a highly significant protective action against autoxidation (Figure 4). Adding phospholipid to the above mixture gave further protection against autoxidation of a dehydrated emulsion. To determine the optimum concentrations of protein and phospholipid for protecting dispersed oil against oxidation several levels of each were studied. As seen in Figure 5, the oxidation rates are significantly decreased by 2% phospholipids (oil basis) and by one part protein to two parts of oil in freeze-dehydrated emulsions.

Effect of phospholipids and phenolic antioxidants on the rate of oxidation of oil emulsions containing iron compounds. It remained now to determine whether phospholipids were also capable of inhibiting iron catalyzed oxidation in these systems. As shown in Figure 6, the onset of hemoglobin catalyzed oxidation was delayed and the extent of this oxidation was reduced by the addition of 5% phospholipid (oil basis) to the emulsion system. The pro-oxidant action of ferric citrate was almost completely nullified by the addition of phospholipid. The protective action of the phospholipid was compared with that of the phenolic antioxidants, BHT and NDGA, at the level of 0.03%. In Figure 7, it is seen that these antioxidants had little effect on decreasing the rate of fat oxidation, whereas phospholipid again showed a significant effect.

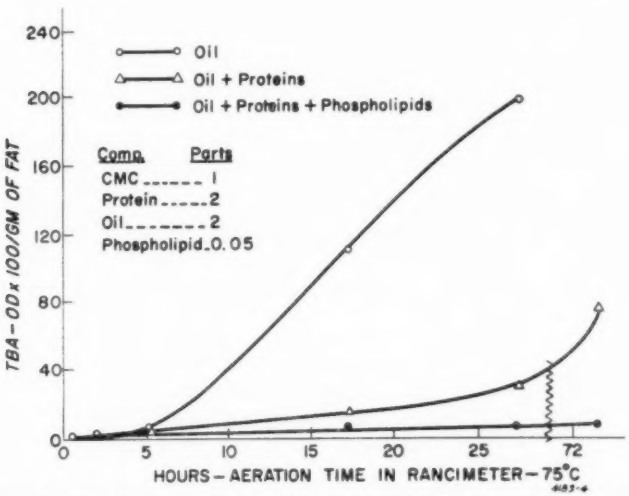

Figure 4. The effects of added proteins and phospholipids on oxidation in dehydrated CMC-oil emulsions.

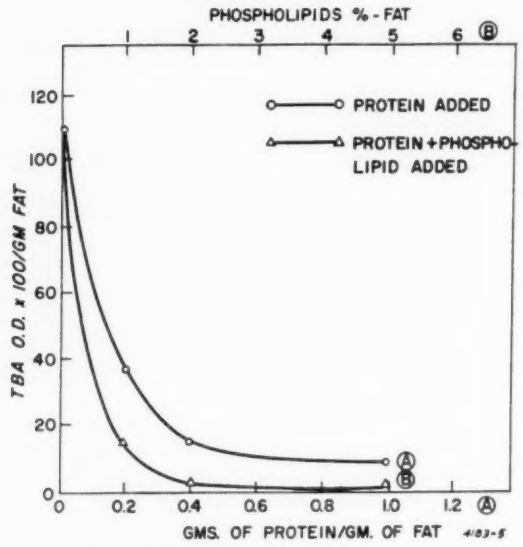

Figure 5. The effects of protein and phospholipid concentration on oxidation of dehydrated emulsions during aeration for seven hours at 75° C.

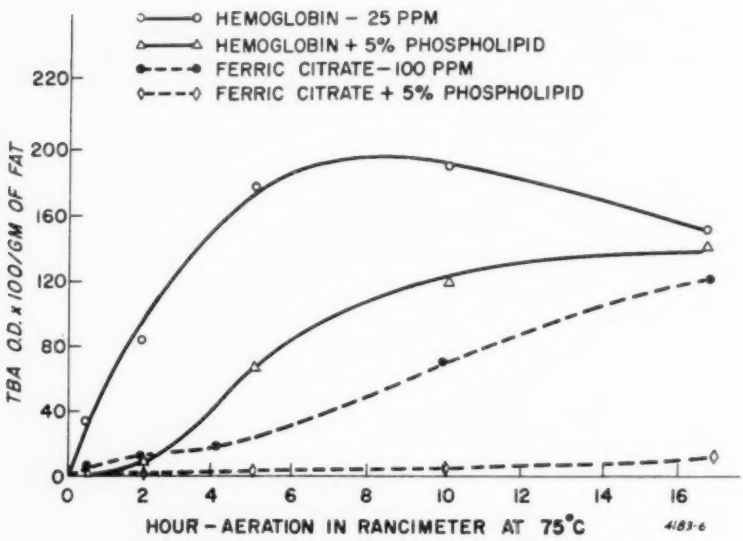

Figure 6. Antioxidant action of added phospholipid in dehydrated emulsions containing CMC, oil and iron compounds. Note: All iron compound concentrations refer to ppm Fe.

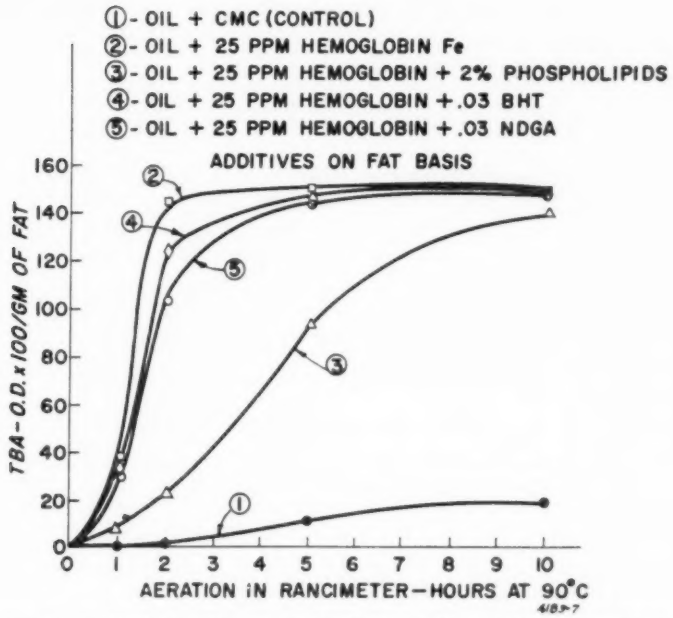
①- OIL + CMC (CONTROL)
②- OIL + 25 PPM HEMOGLOBIN Fe
③- OIL + 25 PPM HEMOGLOBIN + 2% PHOSPHOLIPIDS
④- OIL + 25 PPM HEMOGLOBIN + .03 BHT
⑤- OIL + 25 PPM HEMOGLOBIN + .03 NDGA
ADDITIVES ON FAT BASIS
TBA - O.D. x 100/GM OF FAT
AERATION IN RANCIMETER—HOURS AT 90°C
4/85-7

Figure 7. Antioxidant action of phospholipids, BHT, and NDGA on dehydrated soya oil emulsion containing added hemoglobin.

SUMMARY

Dehydrated fatty emulsions with an inert matrix of carboxymethyl cellulose were used to evaluate the role of proteins, phospholipids and of iron compounds in lipid oxidations under accelerated storage conditions at elevated temperatures. Phospholipids in concentrations of 0.5 to 5.0% of dry weight of fat protected against oxidation. Protein-containing fat emulsions were more stable than those with oil only and in combination with phospholipids protection was even greater. The catalytic effect of hemoglobin on acceleration of the rate of oxidative reaction resulted in spontaneous combustion within minutes in the dried emulsions stored at 85° C. The oxidative reaction rates in dehydrated systems containing porphyrin compounds were significantly more rapid than in fluid systems when measured by peroxides and thiobarbituric acid values. The pro-oxidant effects of ferric citrate were very small compared to those of porphyrin compounds. Phospholipids alone or in combination with proteins had significant antioxidant action in dehydrated emulsions containing porphyrin compounds, while the phenolic antioxidants, BHT and NDGA, at concentrations of up to 0.03% of dry fat weight, had only slight effects. The data presented in this investigation indicate that such naturally occurring food substances as the phospholipids which appear to have antioxygenic properties may find application in attacking the problem of food deterioration caused by fat rancidity.

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THE EFFECT OF SULFUR DIOXIDE AND FERMENTATION ON COLOR EXTRACTION FROM RED GRAPES

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A previous study *(3)* raised questions as to whether the effect of sulfur dioxide on color extraction was different in fermenting musts than in non-fermenting musts, and whether an observed difference in color stability was a varietal characteristic or a function of the concentration of sulfur dioxide. This study also raised a question as to how the color and taste of wines would be affected by fermentation after color extraction. The present study was made to obtain answers to these questions.

EXPERIMENTAL

The grapes used in these studies were grown in the University vineyards at Davis, California, and in commercial vineyards at Lodi.

Must treatment. All experiments were made with 5-gal lots in 20-gal open earthenware crocks. Sufficient individual 5-gal lots were prepared to give the desired number of samples. The entire contents of each crock were used as an individual sample to ensure the same juice-to-pomace ratio for all samples in a given experiment. Uniformity of the lots was ensured by crushing into a 50-gal drum, stirring the contents, and then ladling, during continued stirring, one-quart amounts of must into each crock in turn until 20 additions had been made.

Unless otherwise noted, sulfur dioxide or alcohol was added immediately after the must had been placed in the crock, and each lot was stirred twice daily during the experiment. When fermentation was desired, a 1% culture of the Montrachet strain of *Saccharomyces cerevisiae* var. *ellipsoideus* was added 4 hr later. When fermentation was not desired, 40 ppm was added of captan (*N*-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide), which had been shown *(1)* to have no effect on color. All experiments were conducted at comparable temperatures in the range of 18–26° C.

Sampling and analyses. One-pint samples of the screened 5-gal lots were removed for analysis. The samples of juice were treated and then analyzed with procedures reported previously *(2)*. Since the object was to determine the actual effect of various treatments on color, the color data reported here are those obtained after hydrogen peroxide was added to remove the masking effect of sulfur dioxide.

Taste testing. The wines were tasted by an experienced wine taster at the end of 3 months' storage. Prior to tasting, sucrose was added to bring all the port wines to the same sweetness level. Samples were presented in coded glasses to assure unbiased judgments. The taster was requested to assign numerical scores to the wines, using the usual score card employed in this laboratory.

RESULTS AND DISCUSSION

To simplify presentation of the data the only color characteristic given is brightness (%Y), selected as the best single expression of color density *(2)*. A decrease in %Y indicates an increase in color density.

Effect of sulfur dioxide on fermenting musts. In previous work by the writers *(2)* color stability was found to be about the same in sulfited (100 ppm) and unsulfited Carignane musts, with greater color content in the sulfited must. In Zinfandel musts the one fermented with 250 ppm of sulfur dioxide had the greater initial color, but lost color so rapidly that at the end

of the fermentation its color was only slightly greater than that of the must with 100 ppm, which lost color at a much slower rate. This led to further work with Zinfandel musts *(3)*, in which it was found that, though sulfur dioxide increases color extraction, the color stability is inversely proportional to the concentration of sulfur dioxide. This raised the question whether this difference in color stability was a varietal characteristic of Zinfandel alone, or whether all varieties behaved similarly with increasing concentrations of sulfur dioxide.

The curves in Figure 1 show that sulfited Zinfandel musts are much less color-stable than the corresponding Carignane musts. Other work *(2, 3)* has shown that Cabernet Sauvignon, Petite Sirah, and Valdepenas musts fer-

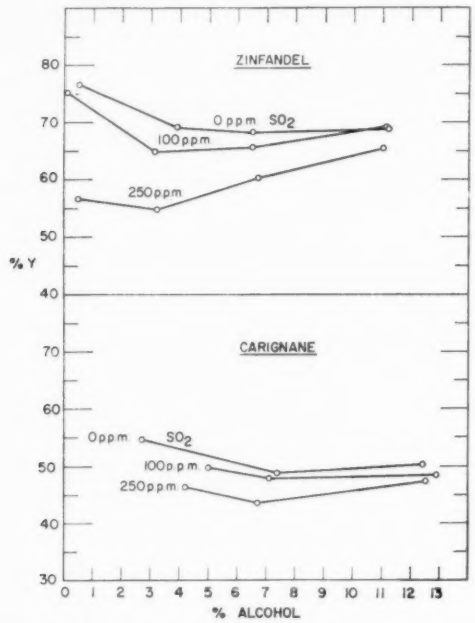

Figure 1. The effect of sulfur dioxide on fermenting musts.

mented with 100 ppm of sulfur dioxide have color stabilities similar to that of Carignane. Thus, we can conclude that color stability is a varietal characteristic, and that Zinfandel is the least stable of the varieties tested.

These curves also show that both grape varieties have greatly decreased color stability at a concentration of 250 ppm of sulfur dioxide. Though more color is extracted at the higher concentration of sulfur dioxide, a greater proportion of the extracted color is unstable, either precipitating or being destroyed in fermentation. Color stability is evidently a function of the concentration of sulfur dioxide, as well as a varietal characteristic.

Effect of sulfur dioxide on non-fermenting musts. In studies in 1957 *(3)* the color stability of unfermented Carignane musts was found to be about the same at 100 and 250 ppm of sulfur dioxide. This led to further work in 1958 to determine if the effect of sulfur dioxide on color stability was different in non-fermenting musts than in fermenting musts.

Two lots of 3 samples each were prepared from a single Zinfandel must and 2 similar lots from a single Carignane must. One lot received 100 ppm of sulfur dioxide and the second lot 250 ppm. Both lots were stored 24 hr at the alcohol concentrations shown in Figure 2. The juice was then separated from the pomace, and all samples of the 2 lots were fortified to about the same alcohol content.

As shown in Figure 2, the color stability of both the Zinfandel and Carignane musts was nearly the same at both levels of sulfur dioxide. It is evident from a comparison of Figures 1 and 2 that the effect of sulfur dioxide is

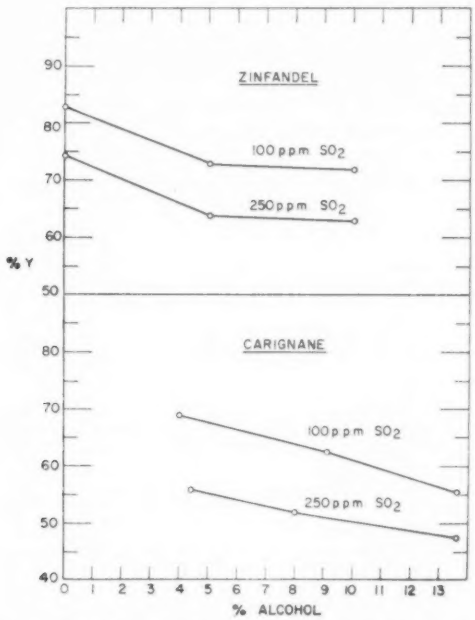

Figure 2. The effect of sulfur dioxide on non-fermenting musts.

different in fermenting musts than in non-fermenting musts, and that fermentation is large responsible for color losses.

Effect of fermentation following color extraction. To test this belief further, 2 lots each of Zinfandel and Carignane wines were made by the procedure given in Table 1. The %Y values plotted in Figure 3 were obtained from readings made on the screened Carignane juices after storage on the pomace for 24 hr following fortification, and on these same juices following

TABLE 1

Experimental procedure in the production of port wines

Treatment	Percent alcohol							
	100 ppm of sulfur dioxide				250 ppm of sulfur dioxide			
	D1	C2	A1	A2	D2	C2	B1	B2
Zinfandel								
Fortified to [1]	0	0	5	10	0	0	5	10
Fermented to [2]	9.6	8.6	11.2	10.0	8.4	8.6	11.5	10.0
Fortified to [3]	19.0	18.3	19.1	19.9	19.0	18.7	19.9	20.0
Carignane								
Fortified to [1]	0	0	5	10	0	0	5	10
Fermented to [2]	13.7	14.3	16.3	14.1	13.7	14.3	16.5	10.5
Fortified to [3]	16.3	16.5	18.5	18.8	16.6	16.5	18.6	19.9

[1] Water was added to the other lots to equal the dilution in lots A2 and B2.
[2] Lots D1 and D2 were fermented on the pomace; the other lots were stored for 24 hours on the pomace, screened, and then fermented for 3 days.
[3] Following fermentation all lots were fortified.

fermentation for 3 days. As shown in Figure 3, fermentation is responsible for a great deal of color loss. These curves also show that the decrease in color is fairly proportional both to the amount of color originally extracted and to the amount of alcohol produced by the fermentation. It is suggested that condensation of pigments with acetaldehyde produced during fermentation is at least partially responsible for the color loss.

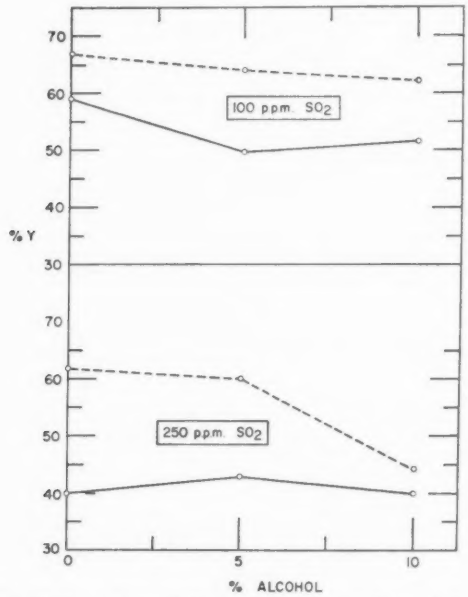

Figure 3. The effect of fermentation. Broken lines—fermented; solid lines—not fermented.

In 1957 *(3)* it was found that more color could be obtained by storing fortified musts for 24 hr than by fermenting the musts to the same alcohol content over a period of 96 hr. Unfortunately, the quality of the wines from the fortified musts was poor. Thus, the experiment reported in Table 1 was performed to determine the effect of fermentation following color extraction not only on color stability but also on the quality of the resultant wines.

As shown in Figure 4, the color content of the wines from the unfer-

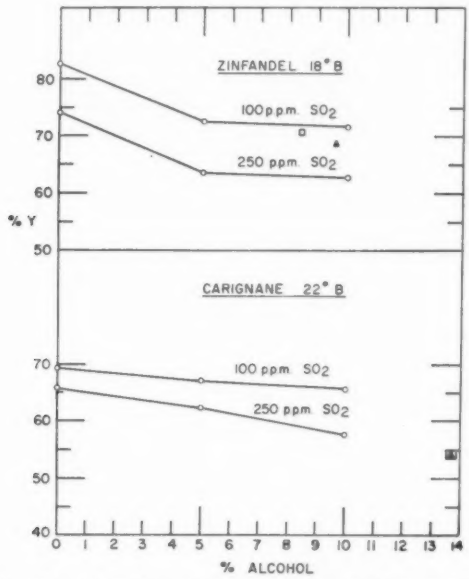

Figure 4. The effect of various must treatments. Solid lines—not fermented on pomace. △—fermented on pomace to alcohol content shown with 100 ppm of sulfur dioxide. □—fermented on pomace to alcohol content shown with 250 ppm of sulfur dioxide.

mented Zinfandel musts at 100 ppm of sulfur dioxide was less, and that at 250 ppm more, than that of the corresponding wines from the fermented musts at the same alcohol level. The wines from both the Carignane unfermented musts contained less color than those from the fermented. Since these color density values were obtained from readings made on the wines after final fortification, they may be compared directly with those of commercial wines. Such comparison shows that even though wines from the Carignane unfermented musts had less color than wines from the fermented musts, they were still well within the range for commercial red wines as reported by Amerine *et al (1)*.

Table 2 shows that fermentation following color extraction produces wines of quality similar to those produced in the conventional manner. Since the color content and quality of the wines were both satisfactory, this procedure is suggested for commercial trial. Savings in labor and capital investment would be very substantial.

TABLE 2

Effect of various must treatments on the quality of port wines

Grape variety	Quality rating							
	100 ppm of sulfur dioxide				250 ppm of sulfur dioxide			
	D1	C1	A1	A2	D2	C2	B1	B2
Zinfandel	74	70	7[illegible]	73	73	72	72	76
Carignane	75	68	72	74	74	71	75	70

However, the procedure has two serious drawbacks: loss of part of the extracted color as a result of the fermentation required to reduce the sugar content to the level found in port, and incomplete color extraction because of the necessity of keeping alcohol content low enough to permit fermentation. To remove these drawbacks, it is suggested that the juice be separated from the pomace immediately after crushing, and fermented to the desired sugar level; that the pomace be fortified to about 20% alcohol content and stored for 24 hr before being screened; that the colored juice be blended with the partially fermented juice and the whole then fortified to 20% alcohol content.

With this procedure, nearly all the color would be extracted from the skins, and color would not be lost as a result of fermentation. Because of this, it is also suggested for a trial to determine which of the 2 experimental procedures is superior.

Effect of fermenting before color extraction. The object of this experiment was to determine if dry red wines of acceptable color and quality could be produced without fermenting on the pomace. Fortification of the must could not be used, for dry red wines, by definition, must contain no sugar and not over 14% alcohol. However, since alcohol is the major factor in color extraction, it was decided to use its effect by adding wine to unfermented pomace.

Zinfandel and Carignane grapes were crushed, 100 ppm of sulfur dioxide added, and the juice immediately separated from the pomace. This juice, which was practically colorless, was then fermented to dryness. Two lots each of Zinfandel and Carignane grapes were then crushed and 100 ppm of sulfur dioxide added. One lot of each, as a control, was then fermented to dryness on the pomace. The other lots were screened immediately, and the nearly colorless wines from the previous fermentations were added to the fresh pomace. After 24 hr these lots were screened, the pomace discarded, and the mixture of juice and wine fermented to dryness.

As shown in Table 3, the Zinfandel had less color, and the Carignane more, than the corresponding control wines produced in the conventional

TABLE 3

Effect of various must treatments on the color content and quality rating of dry red wines

Grape variety	% Y		Quality rating	
	2A2[1]	2A1[2]	2A2[1]	2A1[2]
Zinfandel	58.1	65.0	70	71
Carignane	50.4	45.2	74	73

[1] Fermented on the pomace.
[2] Wine stored for 24 hours on the pomace.

manner. Though the Zinfandel had less color than the control wine, its color was commercially acceptable according to the values given by Amerine *et al (1)*.

Comparison of the quality ratings of the wines shows little difference between the conventional and experimental procedure. Since wines of satisfactory quality and color have been made with this new procedure, a commercial trial seems merited.

SUMMARY

Color stability is a varietal characteristic, with Zinfandel the least stable of the varieties tested. Color stability in fermenting musts is a function of the concentration of sulfur dioxide present.

The effect of sulfur dioxide is different in fermenting than in non-fermenting musts. In the latter, color stability is not affected by sulfur dioxide.

Fermentation is responsible for a great deal of color loss.

Fermentation following color extraction produces wines of color and quality similar to those produced by fermentation on the pomace. The same is true of wines fermented prior to color extraction.

New processes have been suggested for the production of both dry red and port wines.

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THE CAROTENOIDS OF APRICOTS

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Brockmann *(1)* in 1933 reported on the carotenoids of the apricot *(Prunus armenica)*; he used dried fruit and found the main pigment to be β-carotene, with smaller amounts of lycopene and a γ-carotene-like substance. Joyce *(8)* reported that apricots contain, in addition to lycopene, much smaller amounts of polycis pigments. The carotenoids of fresh apricots have now been investigated by use of countercurrent distribution in a Craig appartus to separate the carotenoids into fractions such as hydrocarbons, monols, diols, and polyols, followed by chromatography to separate the individual constituents. This method has been previously applied to the carotenoids of oranges *(3, 6)*, tangerines *(7)*, red grapefruit *(5)*, peaches *(2)*, and leaves *(4)*. β-Carotene was found to be the principal pigment in the apricot. Numerous other carotenoids were present, the ones present in greater amounts being phytoene, phytofluene, γ-carotene, lycopene, cryptoxanthin, and lutein. Both γ-carotene and lycopene were recovered in part as polycis isomers, including pro-γ-carotene.

EXPERIMENTAL

Apricots were obtained at a local market in mid-July, 1955. The fruits selected were ripe, and did not contain green areas or bruised or brown spots. The seeds, which amounted to 10% by weight of the whole fruit, were removed and discarded. The edible portion was divided into 3 lots of (a) 900, (b) 900, and (c) 1900 g, respectively, which were extracted separately.

Each lot was divided into batches of about 250 g, and blended with an equal weight of water to which 6.7 g of calcium carbonate (powder) had been added. The blend was mixed with an equal volume of methanol and, after standing 30 to 45 min, 2% by weight of filter aid was added and the mixture filtered on a Büchner funnel precoated with filter aid. The filter cake was blended briefly with acetone, filtered on a sintered glass funnel, and washed with acetone until the washings appeared colorless. The acetone extract was evaporated *in vacuo* and the lipids removed from the aqueous residue by extraction with ether. Extracts from lots (b) and (c) were saponified *(3)*. The total carotenoid content of the unsaponified and saponified extracts, measured at 440 mmc in an Evelyn colorimeter, was equivalent to 40 and 35 mg of β-carotene, respectively, per kg of the edible portion of fresh fruit.

Countercurrent distribution runs *(3)* of 100 transfers were made in a glass Craig apparatus with both unsaponified and saponified pigments with solvent system I (petroleum ether–99% methanol, 1.8 to 1 by volume). A run of 200 transfers was made with the saponified carotenoids with solvent system II (benzene–petroleum ether–87% methanol, 1 to 1 to 1.15 by volume). The color in each (100-transfer run) or alternate (200 transfers) tubes of the Craig apparatus was measured in an Evelyn photoelectric colorimeter with filter 440, after addition of acetone to make the two phases homogeneous and to a definite volume (25 and 30 ml, respectively, with systems I and II). Results were calculated as β-carotene *(3)* by use of a conversion table.

For the runs with saponified material [from lots (b) and (c)] the contents of the tubes composing each fraction were combined, evaporated *in vacuo*, and the residues dissolved in petroleum ether (hydrocarbons and monols) or benzene, and chromatographed

[a] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

on columns of magnesia (Westvaco No. 2642) plus filter aid (1 to 1 by volume). The bands were fractionally eluted as previously described *(2)*. Spectral absorption curves of the bands so obtained were run in a Cary recording spectrophotometer.

RESULTS AND DISCUSSION

A. Countercurrent distribution with System I. Results of runs with system I for unsaponified and saponified carotenoids are summarized in Table 1.

TABLE 1

Countercurrent distribution of apricot carotenoids with system I (petroleum ether-99% methanol)

Fraction	Percent in carotenoid fraction (measured colorimetrically)		N_{100}[1]	
	Sap	Unsap	Sap	Unsap
I (no free OH)	88.1	96.3	92	91
II A (one free OH)	5.5	2.4	58	56
II B (one free OH + one epoxide group)	0.8	0.4	35	37
III (> one free OH)	5.7	0.9	10	10 and 4

[1] N_{100} = position of maximum calculated on basis of 100 transfers.

The hydrocarbons amounted to about 88% of the total carotenoids. The remainder consisted of about equal parts of monols and diols plus polyols. About 70% of the xanthophylls were originally fully esterified. The minor fraction with an N_{100} value of about 36 probably represents monol epoxides such as cryptoflavin (3-hydroxy-β-carotene-5′,8′ epoxide). The hydrocarbon (I), monol (IIA and IIB combined), and diol-polyol (III-IV) fractions were each chromatographed. The components obtained from the first two are given in Table 2. The components are listed in order of elution, the lowest number first. The diol-polyol fraction was found to be, as in oranges and peaches, very complex and incompletely resolved. Hence another run was made, with solvent system II, in order to further fractionate these carotenoids before chromatography (see below).

Hydrocarbon fraction (I). In the hydrocarbon fraction considerable phytoene (I-1) was found, together with a somewhat smaller amount of phytofluene (I-2); the α-carotene band (I-3) was quite small. The major band was β-carotene (I-4), which, together with a much smaller adjacent band (I-5), apparently a cis-β-carotene, amounted to about 60% of the total carotenoids. The cis-isomers, but not the polycis isomers, may be, at least in part, artifacts formed from the trans-isomers during the experimental procedures. The next band (I-6), which had a greenish fluorescence in ultraviolet light, had spectral absorption maxima close to those of phytofluene and apparently had the same chromophoric system.

Following zeta-carotene (I-7), which is a rather minor constituent, was a series of 8 bands (I-8 to I-15, Table 2) more pink than β-carotene. The spectral absorption maxima and behavior on chromatography of the strongest of these bands (I-10) indicated γ-carotene. A much weaker band eluted just ahead of it (I-9) appeared to be a monocis isomer. The next band (I-8) after zeta-carotene had a spectral absorption curve that was quite different but resembled that of pro-γ-carotene *(10)*, a naturally occurring polycis isomer

TABLE 2

Carotenoid constituents obtained from apricots

Fraction	Constituent[1]	Spectral absorption maxima[2]	Approx %[3]	
I-1	Phytoene	(300), 287, 278	10	
I-2	Phytofluene	366, 347, 332	6.1	
I-3	α-Carotene	472, 443, 416	0.2	
I-4	β-Carotene	477, 450, (427), 338	54	60
I-5	Cis-β-carotene	(470), 446, (424)	5.9	
I-6	Phytofluene-like	369, 346, 333	1.4	
I-7	Zeta-carotene	425, 401, 368, 299, 288	0.6	
I-8	Pro-γ-carotene	454, 434, 348, 336	0.5	4.7
I-9	Cis-γ-carotene	484, 454, 429, 349	0.3	
I-10	γ-Carotene	491, 459, 435, 349	3.9	
I-11	Polycis lycopene a	(465), 440	0.19	4.8
I-12	Polycis lycopene b	469, 446, 347	2.3	
I-13	Polycis lycopene c	(494), 466, (445), 362, (345)	0.4	
I-14	Neo-lycopene B	496, 465, 440, 361, 346	0.9	
I-15	Lycopene	503, 471, 443, 362, (346)	1.0	
II-1	Hydroxy-α-carotene[4]	474, 447, (424)	0.14	
II-2	Cis-cryptoxanthin	470, 445, (423)	0.5	4.0
II-3	Cryptoxanthin	477, 450, (426), 339	3.1	
II-4	Cis-cryptoxanthin	471, 447, (425)	0.4	
II-5	Hydroxy-zeta-carotene[4]	423, 402, (380)	0.3	
II-6	Cis-hydroxy-zeta-carotene[4]	418, 397, (377)	0.2	
II-7	Cryptoflavin[4]	453, 427, 404	0.4	
II-8	Rubixanthin[4]	(486), 454, 433	0.16	
III A-1	Lutein	485, 456, 433, 338	2.1	
III A-2	Zeaxanthin	491, 463, (438), 345	0.7	
III B-1	Carbonyl[4]	421	0.01	
III B-2	Lutein 5,6-epoxide	481, 450, 425, 337	0.02	
III B-3	Antheraxanthin isomer	483, 455, (430), 337	0.06	0.13
III B-4	Cis-antheraxanthin	482, 454, 430, 337	0.07	
III B-5	Flavoxanthin	461, 433, 409	0.03	
III B-6	Mutatoxanthin a	464, 437, (414), 319	0.14	0.23
III B-7	Mutatoxanthin b	465, 439, 415	0.09	
III C-1	Violaxanthin isomer	476, 447, (422), 336, 322	0.09	0.28
III C-2	Cis-violaxanthin	477, 448, 423, 335, 319	0.19	
III C-3	Luteoxanthins	461, 432, 407, 319, 305	0.09	0.49
III C-4	Cis-luteoxanthin a	454, 428, 406, 319, 306	0.2	
III C-5	Cis-luteoxanthin b	455, 428, 404, 318, 305	0.2	
III C-6	Auroxanthins	435, 409, 388, 302	0.3	
IV-1	Persicaxanthin[4]	402, 381, (363)	0.5	
IV-2	Sinensiaxanthin[4]	427, 407, (387)	0.19	
IV-3	Trollixanthin-like	482, 451, 429, 336, 322	0.06	
IV-4	Trollichrome-like	459, 429, 405, 318	0.15	
IV-5	Persicachrome[4]	373, 353, 336	0.09	
IV-6	Cis-trollichrome-like	457, 428, 404	0.05	

Values in parentheses represent shelves or humps on spectral absorption curves.

[1] Cis-isomers (but not polycis or pro-isomers) may be artifacts.

[2] Solvent for fractions I and II was petroleum ether; for IIIA, IIIB, IIIC, and IV, benzene.

[3] Calculated on basis of total absorption of each constituent at its principal spectral absorption maximum.

[4] Tentative identification.

of γ-carotene, which has not been produced artificially. Bands I-8, I-9, and I-10, after iodine isomerization in light *(4)*, had similar spectral absorption curves, as would be expected if they were γ-carotene isomers.

The last band eluted from the column (I-15) had a spectral absorption curve closely resembling that of all-trans lycopene. Fraction I-14 appeared to be a (there are several possible) monocis-lycopene; it was probably neo-lyopene B *(13)*, since the cis-peaks were much too low for neo-lycopene A *(10)*. Fractions I-11, I-12, and I-13 had spectral absorption curves that were quite different from those of I-15 or I-14, but after iodine isomerization they were all similar. Curves for I-11 and I-12 were quite similar to those of polycis lycopenes II and III, respectively, of Zechmeister and Pinckard *(12)*. Shapes of the spectral absorption curves of fraction I-11 to I-13 resembled those of various polycis lycopenes recently obtained by the stereoisomerization of prolycopene, a naturally occurring polycis isomer, by Magoon and Zechmeister *(9)*. Hence prolycopene and/or other polycis lycopenes must occur in the apricot, because polycis lycopenes, including prolycopene, have not been produced artificially from all-trans lycopene *(11)*.

Monol fraction (II). Due to the small amount of carotenoids present, the monol (II-A, Table 2) and monoepoxide monol (II-B) fractions were combined for chromatography. The principal component of the monol fraction was found to be cryptoxanthin. Two minor bands (II-1 and II-7) resembled hydroxy-α-carotene and cryptoflavin, and another (II-8) appeared to be impure rubixanthin (hydroxy-γ-carotene). Two minor bands (II-5 and II-6) were unlike any previously found; spectral absorption maxima were at slightly lower wavelengths than zeta-carotene. Fraction II-6 appeared to be a cis-isomer of II-5, since on iodine isomerization the spectral absorption maxima of the former were increased 2 mmc in wavelength, whole those of the latter were unchanged. These bands apparently were substances with the same number of conjugated double bonds as zeta-carotene, and possibly were hydroxy-zeta-carotenes.

B. Countercurrent distribution with System II. Because the chromatography of the diol-polyol fraction indicated the presence of a very complex mixture, a countercurrent distribution run was made on the saponified carotenoids from a larger quantity of apricots [lot (c)], with solvent system II. In this system the diol-polyol fraction can be separated into 4 parts: diols, monoepoxide diols, diepoxide diols, and polyols *(3, 6)*. The distribution of apricot carotenoids with this system is given in Figure 1. As expected, the hydrocarbon-monol fraction contained well over 90% of the total carotenoids; the diol fraction was somewhat larger than the monoepoxide diol, diepoxide diol, or polyol fractions. Three distinct maxima were apparent in the polyol fraction, probably indicating polyols of somewhat different distribution coefficients (the N_{100} values were 7, 11, and 20). Due to the small amount of polyols present, these were all combined for chromatography. The N_{100} values of the other 4 fractions corresponded closely to those previously obtained with carotenoids from oranges, peaches, and other fruits. The components obtained on chromatography, given in Table 2, are quite similar to those previously obtained from the corresponding fractions of oranges *(6)* and peaches *(2)*.

Diol fraction (III-A). Chromatography of the diol fraction yielded lutein and zeaxanthin only, but with lutein in about 3 times the quantity of zeaxan-

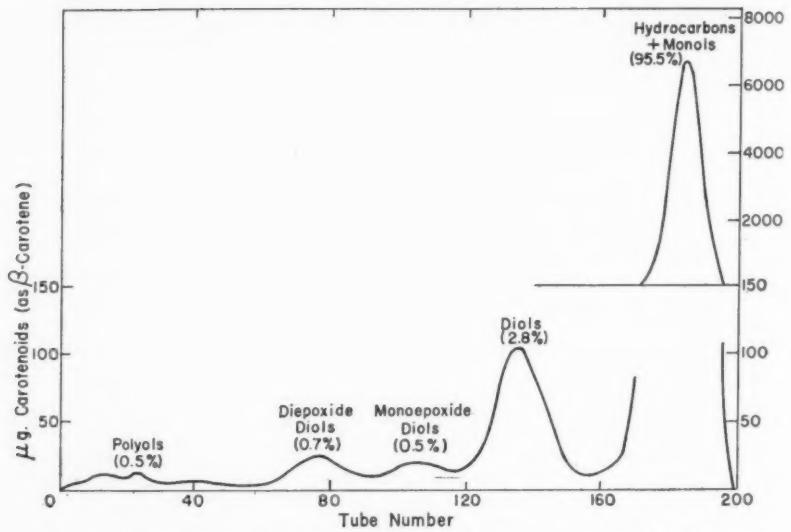

Figure 1. Countercurrent distribution of saponified apricot carotenoids with system II (benzene-petroleum ether-87% methanol).

thin, whereas zeaxanthin was greater in oranges, tangerines, and peaches *(6, 7, 2)*.

Monoepoxide diol fraction (III-B). Constituents obtained from the monoepoxide diol fraction, all in quite small amounts, were very similar to those obtained previously from peaches *(2)*. The spectral absorption curve of III-B-1 had but a single maximum at 412 mmc in benzene; a similar minor fraction had also been obtained from peaches *(2)*. Some carotenoids having a carbonyl group as a part of the conjugated double bond system have curves of similar shape. Two bands were apparently lutein 5,6-epoxide (III-B-2) and the corresponding 5,8-epoxide flavoxanthin (III-B-5). Two pairs of bands were apparently antheraxanthin (zeaxanthin 5,6-epoxide) isomers and mutatoxanthins (the corresponding 5,8-epoxides). The 5,6-epoxides are converted to 5,8-epoxides on contact with acids. In this fraction, the 5,8-epoxides were found in somewhat greater quantities than the corresponding 5,6-epoxides.

Diepoxide diol fraction (III-C). This fraction was found to contain violaxanthin (zeaxanthin 5,6,5′,6′-diepoxide) and the corresponding 5,6,5′,8′-diepoxide (luteoxanthins) and 5,8,5′,8′-diepoxide (auroxanthins) isomers. More luteoxanthins than violaxanthin were found, and about as much auroxanthins as violaxanthin, indicating that here also the 5,6-epoxide-5,8-epoxide isomerization had proceeded to a considerable extent.

Polyol fraction (IV). This fraction on chromatography yielded components which resembled valenciaxanthin, sinensiaxanthin, trollixanthin, trollichrome, and valenciachrome *(6)*. From previous experience with other fruits it is very probable that this fraction was incompletely resolved and was con-

siderably more complex than indicated in Table 2. Further separation and characterization of the polyols was not attempted because of the very small amount of this fraction present. Peaches *(2)* were also found to contain valenciaxanthin-like and valenciachrome-like constituents, which were not identical with valenciaxanthin and valenciachrome from oranges. The names "persicaxanthin" and "persicachrome" were proposed for these components. It seems probable that the substances obtained from apricots were the same as the peach carotenoids rather than those from oranges; they are so designated in Table 2.

In the apricot carotenoids, in 4 instances as noted above, greater amounts of 5,8-epoxides were found than of the corresponding 5,6-epoxides, whereas the opposite was the case in oranges *(6)*, tangerines *(7)*, and cling peaches *(2)*. Similar procedures were used in the extraction of the pigments and in further manipulations. The situation with Elberta peaches *(2)* was similar to that in apricots. The fruit used in these last two were ripe and soft, whereas the cling peaches were quite firm. This suggests the possibility that when acid fruits such as apricots and Elberta peaches become ripe and soft, the acid-catalyzed 5,6-epoxide-5,8-epoxide isomerization may occur to a considerable extent. The above is a further indication that 5,8-epoxides such as auroxanthins, luteoxanthins, flavoxanthin, and mutatoxanthins are not necessarily artifacts, but may occur in nature, as such, in acid fruits. A small amount of 5,6-epoxide-5,8-epoxide isomerization may occur during extraction and subsequent manipulation of carotenoids from plant products, but the ratio of 5,8- to 5,6-epoxides found in the present work was much greater.

SUMMARY

The carotenoids have been examined in a sample of apricots by means of countercurrent distribution followed by chromatography. About 88% of the total carotenoids present were hydrocarbons, with about 6% monols, 3% diols, and smaller amounts of monoepoxide diols, diepoxide diols, and polyols. β-Carotene amounted to about 60% of the total carotenoids. Phytoene, phytofluene, γ-carotene, lycopene, cryptoxanthin, and lutein each accounted for 2% or more of the total carotenoids, while at least 18 other constituents were present in smaller amounts. A part of the γ-carotene and lycopene were recovered as polycis isomers including pro-γ-carotene and 3 polycis lycopenes.

The 5,8-epoxides were obtained in greater quantities than the corresponding 5,6-epoxides, indicating that the 5,6-epoxide-5,8-epoxide isomerization had occurred to a considerable extent during ripening of the apricots.

Acknowledgment

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ASCORBIC ACID STUDIES ON CHILLED, FRESH, AND FERMENTED ORANGE JUICE

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During the course of a survey of the ascorbic acid content of orange juice products sold in Burlington, Vermont, in August, 1955, it was noticed that chilled orange juice in cartons distributed by certain dairies to homes and retail stores was considerably lower in ascorbic acid content than the juice of freshly squeezed oranges purchased locally at the same time. The results were not at all consistent with the description on the orange juice cartons or with advertising claims. In addition, the low reduced ascorbic acid values suggested that considerable oxidation had occurred. This led us to investigate more thoroughly the ascorbic acid content of chilled orange juice sold commercially in the community, including not only determinations of reduced ascorbic acid but also of dehydroascorbic and diketogulonic acids.

EXPERIMENTAL

I. Study of Commercial Chilled Juices

When the study began there were 2 brands of commercial chilled orange juice in cartons available locally. One of the products was straight Florida orange juice and the second consisted of a blend of fresh Florida orange juice and reconstituted frozen Valencia juice. Both products were shipped refrigerated to Burlington. Four months later a third brand, consisting of straight Florida juice shipped refrigerated from Florida, appeared on the Burlington market. For sampling, a carton of each brand was obtained shortly after delivery to homes in each of 3 different sections of the city. Three more cartons of each brand were obtained in this way one week later giving a total of 6 samples for each brand delivered to the home. In addition, a carton of each brand was obtained from each of 3 retail grocery stores in different sections of the city. This was also repeated one week later giving a total of 6 samples of each brand delivered to retail grocery stores. Disregarding the source, the total number of samples of each brand was therefore twelve. Within an hour after being obtained, all samples were being analyzed for ascorbic acid, dehydroascorbic acid, and diketogulonic acid by the 2,4-dinitrophenylhydrazine method of Roe, Mills, Oesterling, and Damron *(5)*. In addition, 2 samples of 6 Florida oranges each, and 2 samples of 6 California oranges each were obtained in local stores. The juice of the 6 oranges comprising each sample was expressed, pooled, and immediately subjected to the above determinations.

II. Study on Refrigeration of Freshly Squeezed Orange Juice

Florida oranges purchased locally were squeezed and the juice pooled. Two-ounce bottles were completely filled with juice, capped, and stored in the refrigerator at 4–5° C. At weekly intervals a bottle of juice was removed from the refrigerator and analyzed for ascorbic acid, dehydroascorbic acid and diketogulonic acid.

III. Fermentation Studies on Fresh Orange Juice

A. To determine whether any relationship existed between fermentation of orange juice and its ascorbic acid concentration, several Florida oranges were squeezed and the juice divided into 3 lots. The first lot was prepared to contain 0.1% baker's yeast initially and, to prevent fermentation, the enzyme poison sodium iodoacetate was added

to make a concentration of 0.001 M. The second lot was prepared to contain the same amount of yeast but no sodium iodoacetate. Nothing was added to the third lot. With each lot, 2-oz bottles were filled, lightly capped to allow any fermentation gases to escape, and stored at room temperature (about 25–27° C) for 7 days during which time periodic analyses for ascorbic, dehydroascorbic, and diketogulonic acids were run on duplicate samples.

B. Bottles of the following solutions were prepared as before: (1) 50 mg% ascorbic acid solution in glass-distilled water, (2) 50 mg% ascorbic acid solution in glass-distilled water and 0.001 M with respect to iodoacetate, (3) orange juice containing initially 0.1% yeast, (4) orange juice containing 0.1% yeast and made 0.001 M with respect to iodoacetate, (5) same as (4) but gassed by the addition of solid CO_2 and capped when under slight CO_2 pressure. On days, 0, 1, 2, 4, and 7, two bottles of each lot were opened, sampled, aliquots titrated for ascorbic acid in triplicate using 2,6-dichlorophenolindophenol *(1)*, and contents of the bottles then discarded.

RESULTS AND DISCUSSION

I. Study of Commercial Chilled Juices

The results are summarized in Table 1. The averaged value for the reduced ascorbic acid content of the 3 chilled orange juice brands was considerably lower than the value of 49 mg% (based largely on reduced ascorbic acid determinations) given for fresh juice in the U. S. Department of Agriculture Handbook No. 8 *(6)* and also lower than the value for juice freshly squeezed from oranges purchased in Burlington. (In January, 1958, ascorbic acid determinations were again carried out on brands A, B, and C. Each contained about 39–40 mg% reduced ascorbic acid, a substantial improvement for brands A and B and no change for brand C which had been 39 mg% originally.) In general, those samples low in reduced ascorbic acid had increased amounts of dehydroascorbic acid [at least 80% active against scurvy *(2)*] and diketogulonic acid (without antiscorbutic activity). In the freshly squeezed juice, on the other hand, the ascorbic acid was found to be present largely in the reduced form in agreement with a previous report *(3)*.

Although the data are not shown, there was remarkable agreement for values of 3 samples of a given brand collected from the stores on the same day. The situation was similar for 3 samples of a given brand collected from homes on the same day. However, the values for similar sets of 3 samples, collected one week later, were generally not consistent with the values of the previous week.

TABLE 1

The ascorbic acid content of commercial chilled orange juice

Brand	Ascorbic acid	Dehydroascorbic acid	Diketogulonic acid
	mg%	*mg%*	*mg%*
A[1]	16.7 ± 8.7 (6–32)	10.5 ± 2.0 (7–14)	11.9 ± 2.9 (7–17)
B[1]	25.8 ± 4.2 (16–30)	4.1 ± 1.9 (1–8)	5.2 ± 1.2 (4–7)
C[1]	39.2 ± 4.4 (31–43)	3.7 ± 1.4 (2–6)	4.3 ± 0.7 (3–5)
Average	27.2 ± 11.2	6.1 ± 3.6	7.1 ± 4.0
Fresh juice[2]	47.4 ± 10.0 (38–60)	1.9 ± 0.8 (1–3)	3.9 ± 3.9 (0.5–8.5)

[1] Values are averages for 12 cartons.
[2] Values are av for 2 samples of 6 California oranges each and 2 samples of 6 Florida oranges each.

TABLE 2

A comparison of the ascorbic acid content of commercial chilled orange juice delivered to stores and to homes

Product	Total ascorbic acid		Reduced ascorbic acid		Dehydro-ascorbic acid		Diketogulonic acid	
	Store[1]	Home[2]	Store	Home	Store	Home	Store	Home
	mg%	*mg%*	*mg%*	*mg%*	*mg%*	*mg%*	*mg%*	*mg%*
A	33.2 ± 0.8 (31–36)	44.3 ± 4.7 (40–50)	9.3 ± 3.0 (6–14)	24.0 ± 5.6 (18–32)	10.0 ± 1.7 (8–12)	11.0 ± 2.4 (7–14)	14.2 ± 1.9 (12–17)	9.5 ± 2.2 (7–12)
B	37.0 ± 1.1 (36–39)	32.7 ± 4.3 (28–38)	28.2 ± 0.4 (27–30)	23.3 ± 5.6 (16–30)	3.3 ± 1.7 (1–5)	4.8 ± 1.9 (3–8)	5.8 ± 1.3 (4–7)	4.5 ± 0.5 (4–5)
C	44.0 ± 4.6 (39–49	50.2 ± 1.5 (48–53)	36.5 ± 4.5 (31–42)	41.8 ± 2.4 (40–43)	3.3 ± 1.2 (2–5)	4.0 ± 1.6 (2–6)	4.0 ± 0.9 (3–5)	4.5 ± 0.5 (4–5)

[1] Average of determinations on 6 cartons.
[2] Average of determinations on 6 cartons.

A comparison of the values of ascorbic acid in cartons delivered to the home and to the store is given in Table 2. There was greater oxidation of ascorbic acid in store-cartons of brands A and C than in home-cartons. The data on brand B indicate that the difference in reduced ascorbic acid between the store and home samples was not significant.

The total ascorbic acid values determined by the dinitrophenylhydrazine procedure can be misleading at first glance to one concerned with the antiscorbutic activity of the orange juice. For example, the average total ascorbic acid concentration of store samples of brand A consisted of only 58% ascorbic acids (reduced ascorbic acid plus dehydroascorbic acid) having antiscorbutic activity. In addition about half of this was dehydroascorbic acid which is unstable, mutarotating to non-antiscorbutic diketogulonic acid *(4)*.

II. Study on Refrigeration of Freshly Squeezed Orange Juice

The results (Fig. 1) are in agreement with those of Mills *et al (3)*. After 2 weeks the juice had still retained 91.5% of its reduced ascorbic acid and 89.0% of its reduced ascorbic plus dehydroascorbic acids. One possibility for increased oxidation of ascorbic acid in the commercially chilled orange juice may be inadequacy of refrigeration. That refrigeration was not always adequate is suggested by the fact that on 5 occasions commercially chilled orange juice was found to be fermented (alcoholic fermentation) on the very day that it was procured. Unexpectedly, the ascorbic acid

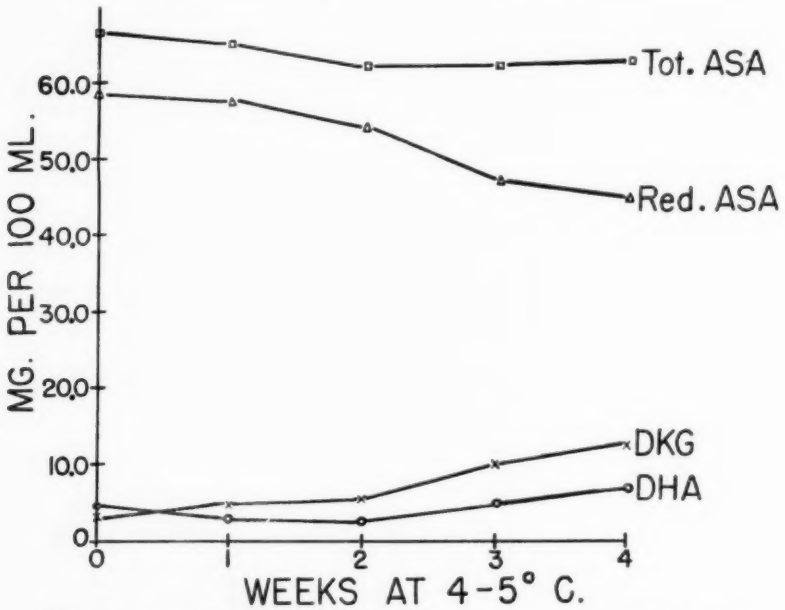

Figure 1. Ascorbic acid values for freshly squeezed Florida orange juice refrigerated for four weeks at 4–5° C. Tot. ASA = total ascorbic acid; Red. ASA = reduced ascorbic acid; DHA = dehydroascorbic acid; DKG = diketogulonic acid.

concentration in these fermented samples was comparatively high while samples with really low values were not fermented.

III. Fermentation Studies on Fresh Orange Juice

The orange juice with added yeast was fermenting (gas formation) vigorously within 8 hours, whereas fermentation was not evident until the second day in the juice without added yeast. No gas formation occurred during the 7-day test in juice containing added iodoacetate. The results are shown in Table 3. After 7 days there was still 80–90% retention of reduced ascorbic acid in the fermented juices whereas there was only 16% retention in the non-fermented juice containing iodoacetate. Several questions arose from this set of experiments. Did iodoacetate oxidize ascorbic acid or catalyze its oxidation? Was the sparing action of fermentation due to reactions that reduce dehydroascorbic acid or was it due simply to oxygen utilization and CO_2 formation?

TABLE 3

Degradation of ascorbic acid in fermented and unfermented orange juice during storage at room temperature

Material		Days stored at 25–27° C				
		0	1[1]	2[1]	4[1]	7[1]
		mg%	*mg%*	*mg%*	*mg%*	*mg%*
Orange juice (fermented)	Ascorbic acid	43.5	40.7	39.4	39.5	37.6
	Dehydroascorbic acid	2.4	2.9	0.4	1.4	1.5
	Diketogulonic acid	3.0	4.7	6.1	6.3	5.8
Orange juice w/added yeast (fermented)	Ascorbic acid	43.5	42.6	41.1	40.0	39.0
	Dehydroascorbic acid	2.4	3.7	0.5	1.4	0
	Diketogulonic acid	3.0	2.5	4.5	5.9	6.5
Orange juice w/added yeast and iodoacetate (unfermented)	Ascorbic acid	43.5	42.3	36.9	20.8	7.0
	Dehydroascorbic acid	2.4	2.5	1.4	3.9	10.4
	Diketogulonic acid	3.0	4.1	8.1	21.0	22.7

[1] Average of determinations on two bottles.

To answer some of these questions another set of experiments was prepared (See II, B of the *Experimental* section). The results are shown in Table 4. Iodoacetate did not enhance the oxidation of ascorbic acid in aqueous solution. The fermented orange juice retained about 87% of its ascorbic acid whereas orange juice plus yeast with iodoacetate to prevent fermentation retained only 31% of its ascorbic acid. The same solution treated with CO_2 retained about 70% of its ascorbic acid.

Although the fermentation studies are to be considered preliminary, the results suggest that the sparing action of fermentation on the ascorbic acid content of orange juice is related to a considerable degree to the utilization or removal of oxygen and its displacement by CO_2 resulting from yeast metabolism and fermentation. Since the sparing action of CO_2 in nonfermented juice was not so great as that occurring in fermented juice, there remains the possibility that, in addition to oxygen removal and CO_2 formation, the reactions of fermentation, particularly those involving hydrogen and electron transfer, act to maintain ascorbic acid in the reduced state. The action of iodoacetate appears to be mainly as an inhibitor of the

TABLE 4

Degradation of ascorbic acid in pure solution and in orange juice stored at room temperature

Test material	Reduced ascorbic acid—mg% Days at 25–27° C				
	0	1[1]	2[1]	4[1]	7[1]
Ascorbic acid	51.1	38.5	35.3	28.6	21.8
Ascorbic acid + Iodoacetate	51.1	41.0	36.2	28.8	28.4
Orange juice + Yeast (fermented)	49.9	50.3	47.7	47.6	43.5
Orange juice + Yeast + Iodoacetate (unfermented)	48.3	48.3	43.2	30.4	15.0
Orange juice + Yeast + Iodoacetate + CO_2 (unfermented)	47.3	49.3	45.1	40.2	33.1

[1] Averages of determinations on two bottles.

fermentation process since it was not found to catalyze the oxidation of ascorbic acid in pure solution.

SUMMARY

The average reduced ascorbic acid concentration of commercial chilled orange juices purchased in Burlington, Vermont in 1955 and 1956 was *27.2* ± 11.2 mg%, only 57% of the value for juice from fresh oranges also purchased locally and 55% of the value for fresh orange juice given in the U. S. Department of Agriculture Handbook No. 8.

Dehydroascorbic acid and diketogulonic acid were found in all samples of chilled orange juice tested. Where reduced ascorbic acid was present in low amounts compared to fresh juice, the amounts of dehydroascorbic acid and diketogulonic acid were relatively high.

A given brand of chilled orange juice delivered to the home did not have the same ascorbic acid content as the identical brand sold in the grocery stores at the same time.

The ascorbic acid content of freshly squeezed orange juice refrigerated at 4–5° C decreased 25% during four weeks and did not increase substantially in oxidized forms until two weeks later.

Fermentation with yeast considerably enhanced the stability of ascorbic acid in orange juice stored at room temperature.

Bubbling CO_2 through orange juice which was protected against fermentation by the addition of iodoacetate had a considerable sparing action on the ascorbic acid present.

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THE STABILITY OF ASCORBIC ACID IN FROZEN AND BOTTLED ACEROLA JUICE ALONE AND COMBINED WITH OTHER FRUIT JUICES[a, b]

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Malpighia punicifolia, Linn., commonly called by its Spanish name "acerola" and known as the West Indian cherry or the Barbados cherry, is a small, bushy tree native to tropical and semitropical regions of America. At present, it is the richest edible fruit source of ascorbic acid reported in the literature.

Asenjo and deGuzman *(1)* first reported that fully ripe cherries grown in Puerto Rico contained from 1030 to 2700 mg of ascorbic acid per 100 g and that unripe fruit might contain as much as 3300 mg/100 g. They also established that the vitamin in the fruit was pure L-ascorbic acid by isolating it in crystalline form. In 1950 Asenjo and Moscoso *(2)* reported additional studies and recommended the use of acerola juice to fortify juice low in ascorbic acid.

In 1952, Mustard *(6)* suggested that the homemaker use acerola juice for jellies, punch bases, and ice cream. Nieva *(7)* discussed the extraction, processing, canning, and keeping quality of acerola juice prepared commercially and noted that heating and canning altered the color and flavor. Losses in ascorbic acid of canned juices stored at room temperature (80 to 85° F) for one year ranged from about 50 to 80% but were only about 20% when stored at 45° F.

This paper reports the results of studies conducted to determine the stability of the ascorbic acid in acerola juice and in three Hawaii fruit juices after fortification with the juice from ripe and half-ripe acerola. The frozen and bottled products were also subjected to organoleptic tests before and after different periods of storage.

The Horticulture Department of the University of Hawaii Agricultural Experiment Station has established test plots of the acerola in Hawaii and has determined that the tree thrives here. Preliminary tests by the Foods and Nutrition Department showed the fruit to contain ascorbic acid within the range of values first reported by Asenjo and deGuzman *(1)*.

Because this fruit may be grown in home gardens of Hawaii or used as a decorative shrub, some basic information on home freezing, canning, and uses of this fruit was needed. Although the methods and results herein reported are for laboratory processes that might be adapted for home use, they are also applicable to commercial processes.

Figure 1 shows the physical characteristics of the fruit which is bright red when fully ripe. The three seeds are large compared to the quantity

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[b] From a thesis by Katherine O. Fitting submitted in partial fulfillment for the degree of Master of Science.

of flesh, but because of their light and pithy nature the seeds constitute only about 20% of the weight. The cherries used in these experiments ranged in size from approximately 3/8 by 1/2 in to 1 3/8 by 1 in and in weight from about 2 to 12 g.

MATERIALS AND METHODS

Preparation of juices. Juice was prepared from both half-ripe and ripe acerola. For present purposes, *ripe* denotes a cherry that is fully red; *half-ripe*, one that is pink or red on less than half its surface and pale green on the remainder. Mixed lots of ripe and of half-ripe acerola from the Department of Horticulture test plots were used. These plants showed variable growth characteristics and some differences in quality of the fruit.

Two types of acerola juice were prepared, hot-pressed and cold-pressed. The hot-pressed juice, which is more practical for the homemaker, was prepared by cooking the fruit with an equal weight of water until soft (5 to 10 min). The mixture was cooled slightly and brought back to the original precooked weight by adding distilled water. Juice was obtained by straining the cooked cherries through 2 thicknesses of cheesecloth.

Cold-pressed juice, though not practical for the homemaker, could be prepared commercially with mechanical equipment. In the laboratory, cold-pressed juice was made by mashing the cherries in an aluminum rotary purée lined with cheesecloth. To bring this juice to essentially the same concentration as the hot-pressed juice, an equal volume of water was added.

No sugar was added to the acerola juice used to fortify other juices.

Hot-pressed acerola juices prepared from ripe and from half-ripe fruit were each equally divided into 2 lots. One lot of each was frozen without sugar, and to the other, sugar was added in a ratio of 1 part sugar to 4 parts juice. Other samples of ripe and half-ripe hot-pressed acerola juice prepared with sugar were bottled. Cold-pressed

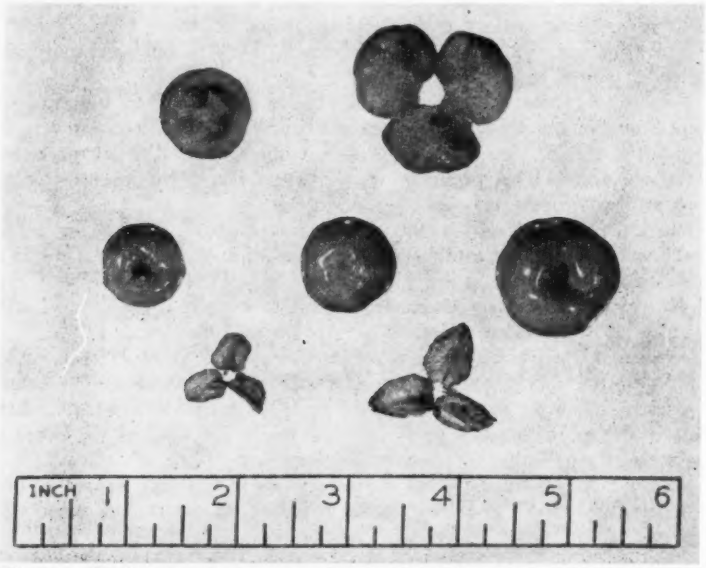

Figure 1. Fruit and seed of acerola. Top, left to right: Cross section of the acerola, longitudinal section of the acerola. Center: Acerola of different sizes showing stem and blossom ends. Bottom: Acerola seeds.

acerola juice samples with and without added water and with sugar in two different proportions were frozen but not bottled.

Fresh pineapple juice was prepared by squeezing the edible portion in 2 thicknesses of cheesecloth. Sugar was added in a ratio of 1 part sugar to 20 parts pineapple juice.

Passion fruit juice was prepared mechanically at the Food Processing Laboratory by use of a paddle pulper. Sugar was added in a ratio of 1 part sugar to 2 parts juice. A similar product can be prepared in the home by cutting the fruit in half, removing the pulp and squeezing it in 2 thicknesses of cheesecloth.

Since fresh juice cannot readily be extracted from ripe guavas, a watery extract was prepared. Sufficient water was added to sliced guavas to almost cover the fruit, which was then boiled for 15 to 20 min. The mixture was poured into a muslin bag and hung up to drain. Sugar was added in a ratio of 1 part sugar to 8 parts juice.

FORTIFICATION OF JUICE FOR STORAGE

Each lot of fruit juice (pineapple, passion fruit, or guava) was divided into 3 equal portions. To one portion, hot-pressed ripe acerola juice was added and to the second, hot-pressed half-ripe acerola juice; the third was stored unfortified as the control.

Because passion fruit juice is usually diluted with water in a ratio of 2 parts water to 1 part juice to make a palatable drink, the amount of acerola juice added was sufficient to make about 50 mg of ascorbic acid per 100 ml in the diluted product.

The Foods and Nutrition Department has previously found that, although good sources of ascorbic acid, both frozen and bottled guava juice lose varying amounts of the vitamin when stored. It seemed worthwhile to determine whether or not acerola juice influenced these losses. The freshly prepared juices were assayed for ascorbic acid and sufficient acerola juice added to bring the concentration of the product as consumed to approximately 60 to 70 mg ascorbic acid per 100 ml.

All juices were frozen and bottled in sufficient quantities for ascorbic acid determinations and organoleptic testing.

STORAGE OF JUICE

Frozen juice. Prepared juices were stored in pint plastic bags. After pressing out the air and sealing, the bags were placed inside moisture vapor proof cardboard containers. The smaller quantities of acerola juices were frozen in aluminum freezer trays. Each cube was then covered with "Saran wrap" and all cubes of like kind were placed in plastic bags for storage.

Bottled juice. Small lots of juice were boiled for 3 min, poured into hot sterilized, 8 oz, dark green bottles and capped with metal, cork-lined bottle caps. Bottles were stored away from light at room temperature.

TEST PERIODS

Ascorbic acid contents of all juices were determined after 0, 3, 6, and 8 months of storage. Two frozen packages and 2 bottles of each type of juice were tested at each period. During the early months of the experiment, the ascorbic acid values of any 2 duplicate samples did not vary more than 2% in juice with ascorbic acid over 20 mg/ 100 ml and did not vary more than 4% in juice with ascorbic acid under 10 mg/ 100 ml; mean values for the 2 samples are reported for each test period. In the later months of the experiment, some samples above 20 mg/100 ml varied as much as 4%.

DETERMINATION OF ASCORBIC ACID

Because the juices were colored, the photometric method of determining the reduced form of ascorbic acid as described by Loeffler and Ponting *(4)* and *Methods of Vitamin Assay (3)* using the Evelyn photoelectric colorimeter was employed. Two frozen and 2 bottled samples of each type of juice were tested at each period.

RESULTS AND DISCUSSION

Acerola juice. Seven lots of ripe and 7 of half-ripe acerola, varying in weight from less than 1 lb to more than 2 lb each, were used over a period

of 2 months for 14 lots of hot-pressed juice prepared in the manner described above. The quantity of juice extracted averaged 48% for the ripe acerola and 35% for the half-ripe. The ascorbic acid content of the ripe juice varied from 1010 to 1240 mg with an average of 1100 mg/100 ml and the half-ripe juice showed a range of 1160 to 1370, with an average of 1250 mg/100 ml. The pH of each lot was not determined but several samples of hot-pressed acerola juice averaged 3.3.

Results of the storage tests are summarized in Table 1 and Figure 2. All the frozen acerola juice whether hot-pressed or cold-pressed and whether from ripe or half-ripe cherries retained approximately the same percentage (82 to 87%) of ascorbic acid at the end of an 8-month storage period. The bottled product, on the other hand, retained only about 60% of the original ascorbic acid at the end of a similar storage period. Neither diluting the

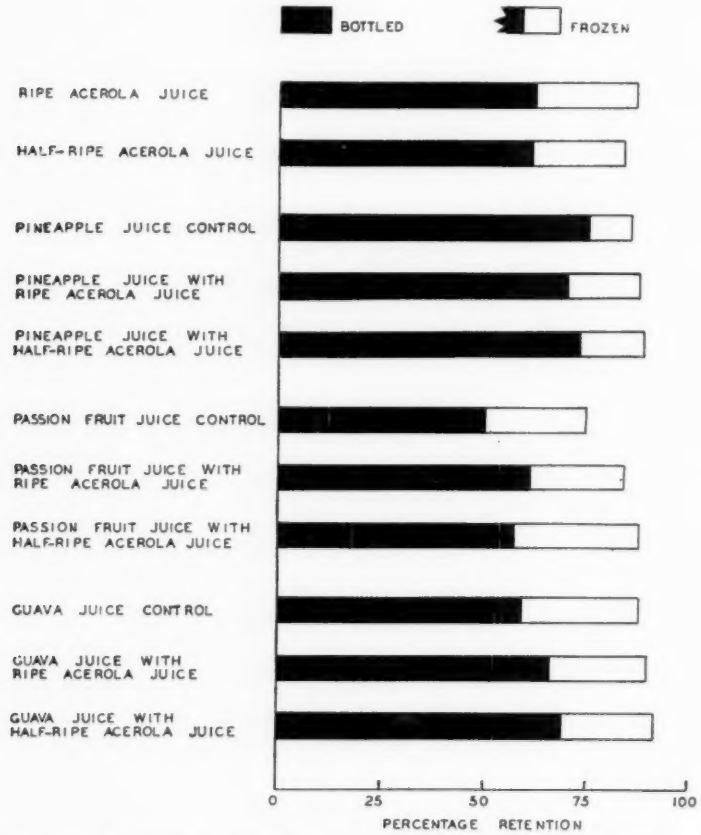

Figure 2. Retention of ascorbic acid in bottled and frozen juices after 8 months' storage.

TABLE 1

Ascorbic acid retention in frozen and bottled acerola juice with and without sugar

Method of storage and type of juice	0 months	3 months		6 months		8 months	
	Ascorbic acid	Ascorbic acid	Retention	Ascorbic acid	Retention	Ascorbic acid	Retention
	mg/100 ml	*mg/100 ml*	%	*mg/100 ml*	%	*mg/100 ml*	%
Acerola Juice (hot-pressed)							
Frozen							
Ripe without sugar	1105	999	90	945	86	925	84
Ripe with sugar in 1 : 4 ratio	855	819	96	773	90	745	87
Half-ripe without sugar	1215	1095	90	1034	85	981	82
Half-ripe with sugar in 1 : 4 ratio	940	856	91	805	86	791	84
Bottled							
Ripe with sugar in 1 : 4 ratio	875	688	79	626	72	540	62
Half-ripe with sugar in 1 : 4 ratio	1180	919	78	805	68	720	61
Acerola Juice (cold-pressed)							
Frozen							
Pure juice, sugar in 1 : 4 ratio	1312	1180	90	1105	84	1101	84
1 : 1 water-juice, sugar in 1 : 4 ratio	796	756	95	718	90	654	82
1 : 1 water-juice, sugar in 1 : 3 ratio	775	744	96	731	93	660	85

juice with water nor adding sugar appeared to affect the stability of the ascorbic acid in the frozen product.

Pineapple juice. The stability of the ascorbic acid in pineapple juice fortified with juice from ripe and half-ripe acerola is illustrated in Table 2 and Figure 2. There was almost 90% retention of the ascorbic acid in the frozen fortified juices and about 70% in the bottled juices after storage for 8 months.

Passion fruit juice. Results of tests on the stability of ascorbic acid in passion fruit juice frozen and bottled after fortification with juice from ripe and half-ripe acerola are shown in Table 2 and Figure 2. Undiluted passion fruit juice has a very strong flavor, and, as previously noted, it was fortified with acerola juice to the extent that when diluted with 2 parts water to 1 of juice the product would have approximately 50 mg ascorbic acid/100 ml.

Retention of the ascorbic acid (84 and 88%) in the fortified frozen passion fruit juices was similar to that for pineapple, but the bottled juices retained only about 60% or approximately 10% less than the bottled pineapple juice.

Guava juice. Work in this laboratory had previously indicated that bottled guava juice (a watery extract as previously described) retained only 70% of the original ascorbic acid after 11 months of storage despite its high acidity (pH 3.5) *(5)*. In a later set of experiments guava juice prepared in the same manner showed a retention of 80 to 85% of the ascorbic acid after 12 months' freezer-storage *(8)*.

Although the period of storage was less for the experiments reported here, results shown in Table 2 and Figure 2 demonstrate clearly the superior retention of ascorbic acid in the frozen product (90%) as compared with the bottled (67%). Additional experiments would be required to determine whether or not acerola juice will reduce the rate of loss in guava juice.

All of the fortified juices retained ascorbic acid to about the same degree after 8 months' storage.

EFFECT OF FREEZING AND BOTTLING UPON THE COLOR AND FLAVOR OF ACEROLA JUICE AND OF OTHER JUICES FORTIFIED WITH ACEROLA

Acerola juice. The color of the juice when first prepared was an attractive pink. After 2 weeks of frozen storage it had developed an orange tint, which it retained for the remainder of the storage period. The color of the bottled juice changed from a pale pink to amber by the end of the 3-month storage period but did not seem to change thereafter. The half-ripe juice had less color than that from ripe cherries.

After thawing, colored particles precipitated in both ripe and half-ripe juice stored without sugar, forming a layer of liquid with high chroma on the bottom and a pale layer on top. The color of the ripe and half-ripe juices stored with sugar remained homogeneous, indicating that sugar had a stabilizing effect. The cold-pressed juice which was frozen with sugar retained color somewhat better than the hot-pressed. That which was bottled changed to the same amber color as the bottled hot-pressed juice.

TABLE 2

Ascorbic acid retention in frozen and bottled juices when stored alone and when fortified with ripe or half-ripe acerola juice

Method of storage and type of juice	0 months	3 months		6 months		8 months	
	Ascorbic acid	Ascorbic acid	Retention	Ascorbic acid	Retention	Ascorbic acid	Retention
	mg/100 ml	*mg/100 ml*	%	*mg/100 ml*	%	*mg/100 ml*	%
Pineapple Juice							
Frozen							
Control	7	6	86	6	86	6	86
With ripe acerola juice	59	56	95	53	90	52	88
With half-ripe acerola juice	55	54	98	49	89	49	89
Bottled							
Control	8	7	87	6	75	6	75
With ripe acerola juice	60	54	90	46	77	42	70
With half-ripe acerola juice	63	60	95	50	79	46	73
Passion Fruit Juice							
Frozen							
Control	8	6	75	6	75	6	75
With ripe acerola juice	157	142	90	136	87	132	84
With half-ripe acerola juice	151	144	95	136	89	134	88
Bottled							
Control	8	6	75	5	63	4	50
With ripe acerola juice	171	127	74	111	65	105	61
With half-ripe acerola juice	178	119	67	109	61	101	57
Guava Juice							
Frozen							
Control	67	61	91	60	90	59	88
With ripe acerola juice	76	70	92	69	91	68	90
With half-ripe acerola juice	72	69	96	68	94	66	92
Bottled							
Control	54	42	78	33	61	32	59
With ripe acerola juice	71	59	83	50	70	47	66
With half-ripe acerola juice	65	56	86	48	74	45	69

There was no perceptible change in flavor of the frozen or bottled acerola juices after storage for 8 months.

Fortified juices. A taste panel rated the frozen and bottled fortified juices after 0, 3, 6, and 8 months of storage. Three samples, identified only by number, which contained juice fortified with ripe acerola juice, juice fortified with half-ripe acerola juice and unfortified juice were placed before the panel members. The panel was instructed to compare the three numbered samples with a control sample of unfortified juice for flavor and color, and to evaluate the numbered samples on the basis of an over-all or general impression.

Fortification of frozen pineapple juice, passion fruit juice, and guava juice with ripe and with half-ripe acerola juice had little if any effect on flavor and color of juice throughout an 8-month storage period.

Fortification of bottled (heated) pineapple juice and guava juice with ripe and with half-ripe acerola juice likewise had little if any effect on flavor and color of juice. All samples of bottled (heated) passion fruit juice showed only slight changes in color, but deteriorated markedly in flavor during storage to the extent that they were definitely unpalatable.

SUMMARY AND CONCLUSIONS

The acerola (*Malphigia punicifolia,* Linn.), also known as the West Indian or Barbados cherry, is generally acknowledged to be the richest edible fruit source of ascorbic acid, ca 2000 mg/100 g.

Juices, prepared in the laboratory, which contained approximately 1200 mg ascorbic acid per 100 ml were frozen and bottled with and without sugar. Unsweetened acerola juice was used to enhance or fortify three tropical fruit juices—pineapple, passion fruit, and guava—which were then frozen or bottled.

After storage for 8 months all frozen juices retained similar proportions of ascorbic acid, 82 to 92%. All bottled juices retained consistently less ascorbic acid, approximately 60 to 70%.

Acerola juice had little or no effect upon the color or flavor of the fortified juices. Sugar appeared to have some stabilizing effect upon the color of frozen acerola juice.

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COMPOSITION OF SOME SOUTH PACIFIC FOODS

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Nutritional problems of the South Pacific region are extremely complex and differ widely within the area. The low atolls, the high islands, and the large land masses of the New Guinea–Solomons Archipelago each have their own peculiar set of problems.

Since the end of the war, concentrated efforts have been made by different organizations to investigate some of these problems. In particular, the Australian Government organized a full-scale nutritional survey in Papua–New Guinea in 1947 *(9)* and two international bodies—the South Pacific Health Service and the South Pacific Commission—set up nutrition sections and have carried out nutrition surveys of many Pacific territories.

As part of the South Pacific Commission's nutritional and subsistence economy programs, a laboratory was established in 1954 in Noumea, New Caledonia, to determine the proximate composition of local foods being used by the Pacific peoples. This work was extended in 1956 to include certain vitamin analyses, and a start was made to determine the amino acid content of the foods. The laboratory was closed because of a lack of funds at the end of 1956. A general report of the work has been published *(18)*.

A survey of the literature *(17)* showed that very little had been published on the composition of South Pacific foods and it is hoped that this report helps to fill in some of the blanks.

EXPERIMENTAL

Methods of analysis. Moisture was determined in all the food samples by the entrainment distillation method using toluene as the distilling agent *(24)*.

The method of van de Kamer and van Ginkel *(23)* was used for the determination of crude fiber. It gave reproducible results comparable with those obtained by the acid-alkaline digestion method and was much simpler to use than this latter method.

Crude fat was determined by the Soxhlet extraction method using di-isopropyl ether as the solvent. Because of the consistently high daily air temperatures, up to 38° C, di-isopropyl ether (B.P. 68.5° C) was found to be more satisfactory than diethyl ether. The ash was determined by ignition of the residue from the ether extraction at 600° C in a muffle furnace.

Total nitrogen was determined using the Conway *(7)* micro-diffusion method.

Soluble reducing sugars were determined using the ethyl alcohol extraction method of the Association of Official Agricultural Chemists *(3)*. Crude starch was determined by hydrolyzing the residue left after alcoholic extraction with hydrochloric acid.

Calcium was determined by the oxalate precipitation method followed by titration with potassium permanganate.

Phosphorus was determined volumetrically by the AOAC method.

Carotene was determined by the chromatographic method published in *Methods of Vitamin Assay (4)*. The eluted bands were measured in the range 400–500 mmc on a "Unicam" SP500 spectrophotometer and the results expressed as β-carotene, although α-carotene and neo-isomers of β-carotene may have been present. Activated

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alumina, type H, was used as the adsorbent. The eluent consisted of 3% acetone in 65°–70° C petroleum ether. Eastman-Kodak carotene containing 20% α- and 80% β-carotene was used for the standard calibration curve.

For thiamine determinations the samples were digested and purified according to *Methods of Vitamin Assay (4)* and the thiochrome was prepared and determined using the modified method of the Kraft Foods Ltd. laboratory in Melbourne, Australia. This modification consists of first extracting the acid potassium chloride eluent with water saturated *n*-butanol to remove some of the interfering substances. The thiochrome was then developed by adding 1 drop of 2% potassium ferricyanide to the thiamine solution to which has been added alkaline methanol. The thiochrome is extracted into glass redistilled *n*-butanol and read on a fluorimeter.

Riboflavin analyses were not made as the filters necessary for the fluorometric method were not received until after the laboratory had been closed.

The chemical method recommended by the *Methods of Vitamin Assay (4)* was generally followed for niacin determinations. However, because of the small amounts of niacin present in most of the plants tested, the method was changed so that 10 g of sample was hydrolyzed with 15 ml 1ON HCl and 45 ml of water for at least 8 hr, filtered and all of the filtrate used for adsorption on Lloyd's reagent.

The 4-methoxy-2-nitroaniline method of Schmall *et al (21)* was used for determining ascorbic acid in coloured solutions and the 2,6-dichlorophenol indophenol visual titration method was used for colourless solutions *(3)*.

Amino acids were determined on a 150 cm Dowex 50 x 8% column using the method of Moore and Stein *(12)*. Cystine and methionine were determined after performic acid oxidation as described by Schram *et al (22)*.

Collection of samples. The samples examined came from widely scattered areas of the Pacific and were flown to Noumea. The samples were collected by territorial agricultural or medical officers or by Commission personnel visiting the areas. The procedure used for the preservation of the samples during shipment was based on that described by Munsell *et al (14)*. In most cases Waring blenders were not available and the samples were very finely chopped with a knife.

Each collector was provided with a set of instructions, data sheets for recording weight of food used and geographical background and sealed ampules containing 2 g of oxalic acid crystals. Two samplings were prepared, one consisting of 400 g or 14 oz of the food with 2 g oxalic acid and 200 ml water and the other consisting of 50 g or 2 oz of the food with 2 g oxalic acid and 200 ml water. Except for samples of 2 leaves from Papua, carotene determinations were made only on fresh samples obtained in New Caledonia or on uncut tubers or Pandanus fruit. These leaf samples were preserved by the method described by Munsell *et al (14)* for carotene samples. Tubers were forwarded uncut and legumes were sent dry.

Photographs, leaves and flowers were also collected for botanical identification of the specimens. The origin of the various samples is shown in Table 1.

RESULTS AND DISCUSSION

A full description of the various plants and the ways in which they are used is given by Barrau *(5, 6)* and Massal and Barrau *(10)*.

Results obtained for the proximate composition and vitamin content are given in Table 2. For ease of interpretation these results are divided into (a) roots and tubers, (b) starches, (c) fruits, (d) nuts, (e) leaves, (f) saps and juices, (g) others. The amino acid analyses of taro and yams are given in Table 3.

TABLE 1

Origin of samples

Botanical name	Common name	Origin
Artocarpus altilis	Bread fruit	
	fresh	Apia, Western Samoa
	smoked	Reef Is., British Solomon Is. Protectorate
Bambusa sp.	Bamboo shoots	Mandum, Netherlands New Guinea
Bruguiera eriopetala	Aibon (mangrove fruit preparation)	Biak, Netherlands New Guinea
Cajanus pseudocajan	Pigeon Pea	Oundjo, New Caledonia
Carica papaya	Pawpaw (papaya)	
	fruit green	Noumea, New Caledonia
	ripe	Noumea, New Caledonia
	leaves	Muting, Netherlands New Guinea & Noumea, New Caledonia
Cocos nucifera	Coconut	
	palm sap	Biak, Netherlands New Guinea
	mature meat	Noumea, New Caledonia
	green meat	Noumea, New Caledonia
	water	Noumea, New Caledonia
	"apple"	Noumea, New Caledonia
Colocasia antiquorum	Taro	Noumea, New Caledonia
Colocasia esculenta	Taro	
	tubers	Tie, New Caledonia
	leaves	Noumea, New Caledonia
Cucurbita pepo	Pumpkin leaves	Goilala, Papua
Cyrtosperma chamissonis	Taro	Santo, New Hebrides
Dammaropsis kingiana	"leaves"	Goilala, Papua
Dioscorea aleta	Yam	
	white	Various districts, New Caledonia
	red	Mare, Loyalty Is.
Dioscorea pentaphylla	Yam	
	green	Ateu, New Caledonia
	purple	Ateu, New Caledonia
Eugenia jambos	Jambo, Eugenia fruit	Netherlands New Guinea
Ficus sp.	Fig	Ateu, New Caledonia
Gnetum gnemon	Gnemon leaves	Marouke, Netherlands New Guinea
Hibiscus manihot	Hibiscus leaves	Tie, New Caledonia
Inocarpus edulis	Polynesian chestnut	Apia, Western Samoa, Sigatoka, Fiji
Ipomoea batatas	Sweet potato	
	yellow	Noumea, New Caledonia
	white	Noumea, New Caledonia
		Wissel Lakes, Netherlands New Guinea

TABLE 1 (continued)

Botanical name	Common name	Origin
Lumnitzera racemosa	Mangrove leaves	Oundjo, New Caledonia
Manihot dulcis	"sweet" cassava	
	tubers	Noumea, New Caledonia
	leaves	Noumea, New Caledonia
Metroxylon sagu	Sago and sago preparations	Muting, Netherlands New Guinea
	Sago boengkoes	
	Sago bulu	Muting, Netherlands New Guinea
	Sago ega	Biak, Netherlands New Guinea
	Sago lempeng	Mandum, Netherlands New Guinea
	Raw sago	Mandum, Netherlands New Guinea
	Sago senole	Biak, Netherlands New Guinea
	Sago sep	Biak, Netherlands New Guinea
Musa pardisiaca var. *sapientum oleracea*	Banana rhizome	Lifou, Loyalty Is.
Nipah fruticans	Nipa	Waropen, Netherlands New Guinea
	palm sap	
Oryza sativa	Rice	
	Imported and Fijian	Suva, Fiji
Pandanus julienetti	Pandanus	
	"nuts", smoked	Goilala, Papua
Pandanus sp.	Pandanus	Marita, Papua
Pandanus sp.	Pandanus	Noumea, New Caledonia
Pandanus sp.	Pandanus	Tarawa, Gilbert and Ellice Islands colony
Phascolus lunatus	Bean	Goilala, Papua
Pteris moluccana	Fern root flour	Fredrik Hendrik Is., Netherlands New Guinea
Pueraria thunbergiana	Magnagna, Kudzu	Ateu, New Caledonia
Sesbania grandiflora	Sesban	
	leaves	Noumea, New Caledonia
	flowers	Noumea, New Caledonia
Solanum sp.	tuber	Goilala, Papua
Tacca leontopetaloides	Polynesian arrowroot	Tuamotu Is., French Oceania
Tetragonia expansa	New Zealand spinach	Kone, New Caledonia
Wedelia biflora	Leaves	Tie, New Caledonia
Xanthosoma sagittifolium	Taro	Noumea, New Caledonia
? ? ?	Fungus	Goilala, Papua
? ? ?	Dried Fish	Malaita, British Solomon Islands Protectorate

TABLE 2

Proximate and vitamin composition

Food	No. samples	Water	Ash	Fibre	Ether extract	Total nitrogen	Reducing sugars	Starch	Carotene	Thiamine	Niacin	Ascorbic acid
		%	%	%	%	%	%	%	mgm%	mgm%	mgm%	mgm%
(a) Starchy roots												
Colocasia antiquorum	7	59.0	1.3	1.0	0.5	0.120	0.2	35.0	...	...	...	...
Colocasia esculenta	5	53.5	0.9	3.0	0.5	0.230	0.5	35.5	0.003	0.270	0.202	5
Cyrtosperma chamissonis	3	60.0	1.0	1.0	0.5	0.155	0.5	33.0	0.008	0.319	0.059	3
Dioscorea alata												
white	13	54.5	0.5	0.5	0.1	0.270	0.2	38.0	nil	0.145	0.191	trace–8
red	8	68.0	0.4	0.6	0.1	0.344	0.1	30.5	0.132	0.125	0.297	4–8
Dioscorea pentaphylla												
green	2	83.0	0.2	0.7	2.0	0.230	nil	14.0	0.556	0.061		trace
purple	2	75.0	0.1	0.7	4.0	0.160	1.5	16.5	0.720	0.048	0.099	...
Ipomoea batatas												
white	10	68.0	0.7	0.6	0.1	0.130	0.5	28.0	0.036	0.065	0.700	62
yellow	8	68.0	0.9	0.8	0.1	0.100	0.5	29.5	0.149	0.077	0.242	68
Manihot dulcis—tubers	5	60.0	0.6	1.0	0.7	0.060	8.0	31.0	nil	0.038	0.279	26
Musa paradisiaca var. *sapientum oleracea*	1	70.5	0.4	1.0	0.5	0.180	10.0	18.5	0.064	0.028	0.198	trace
Pueraria thunbergiana	2	63.0	1.5	5.2	2.2	0.215	6.0	21.5	nil		0.243	1
Solanum sp.	1	79.0	0.9	2.4	6.0	0.510	nil	5.0	3.990	0.026	0.747	...
Xanthosoma sagittifolium	5	59.0	1.5	1.0	0.5	0.075	0.2	34.5	trace	0.258	0.329	6
(b) Starches												
Artocarpus altilis												
fresh	2	80.0	0.3	1.2	1.5	0.150	10.5	13.5	...	...	...	...
smoked	1	8.0	4.0	4.5	2.0	0.680	nil	72.0	...	0.080	...	72
Bruguiera eriopetala												
paste	1	72.0	3.0	4.0	0.5	0.402	nil	16.0	...	...	...	...
Metroxylon sagu												
raw sago	11	27.0	0.1	0.3	nil	0.035	nil	71.0	...	...	...	...
sago boengkoes	8	42.0	0.5	0.5	2.0	trace	0.2	53.0	...	...	...	...

TABLE 2 (continued)

Food	No. samples	Water	Ash	Fibre	Ether extract	Total nitrogen	Reducing sugars	Starch	Carotene	Thiamine	Niacin	Ascorbic acid
		%	%	%	%	%	%	%	mgm%	mgm%	mgm%	mgm%
sago bulu	8	55.0	1.5	1.0	1.0	trace	1.0	39.0	.....	.....	.....	....
sago ega	6	37.5	0.5	1.8	nil	0.010	0.5	53.0	.....	.....	.....	....
sago ega	5	52.5	0.5	0.6	nil	trace	0.5	40.0	.....	.....	.....	....
sago lempeng	12	12.0	0.3	0.2	nil	trace	nil	84.0	.....	.....	.....	....
sago senole	10	23.0	0.5	1.0	16.5	0.300	0.5	59.0	.....	.....	.....	....
sago sep.	8	16.5	0.5	1.5	nil	0.098	nil	79.5	.....	.....	.....	....
Pteris moluccana												
flour	2	13.0	1.0	10.0	nil	2.000	0.2	67.0	.....	.....	.....	....
Tacca leontopetaloides												
flour	1	18.0	....	0.05	3.0	0.010	nil	76.0	.....	.....	.....	....
(c) Fruits												
Carica papaya												
green	5	91.0	0.5	0.5	0.1	0.128	2.5	3.5	.....	.....	.....	....
ripe	8	88.0	0.1	0.5	0.1	0.095	5.5	2.5	.....	.....	.....	....
Eugenia jambos	2	88.5	0.01	0.7	5.0	0.032	2.0	0.5	.....	.....	.....	....
Ficus sp.	1	72.0	1.9	7.0	3.2	0.355	4.6	5.7	0.975	0.079	0.401	2
Pandanus sp.												
Gilbert Is.	3	70.0	0.5	1.0	2.5	0.980	7.2	13.0	0.011	0.034	.....	5
New Caledonia	1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	8–18
New Guinea	2	67.0	0.5	12.6	14.0	0.590	nil	6.7	.....	0.140	1.372	....
Pandanus julienetti												
smoked	2	4.0	2.5	22.0	59.0	1.720	2.3	3.5	.....	0.225	1.310	....
(d) Nuts												
Cocos nucifera												
mature meat	15	50.5	1.0	4.2	30.8	0.641	nil	12.0	.....	0.085	.....	4
green meat	16	56.2	0.8	2.5	27.8	0.721	4.6	4.2	.....	.....	.....	....
"apple"	12	87.0	1.1	1.2	2.7	0.173	6.4	0.5	.....	.....	.....	4
Inocarpus edulis	3	43.0	1.5	2.5	4.5	0.725	9.5	31.0	nil	0.261	.....	2

TABLE 2 (continued)

Food	No. samples	Water	Ash	Fibre	Ether extract	Total nitrogen	Reducing sugars	Starch	Carotene	Thiamine	Niacin	Ascorbic acid
		%	%	%	%	%	%	%	mgm%	mgm%	mgm%	mgm%
(e) Leaves												
Bambusa sp.	1	79.0	0.3	3.0	3.0	0.420	3.0	1.5	.....	.....	.....	....
Carica papaya	5	75.0	2.5	2.0	2.0	1.255	2.5	6.0	.....	.....	.....	....
Colocasia esculenta	4	85.0	2.0	2.3	2.5	0.801	2.8	1.5	.....	.....	.....	....
Cucurbita pepo	1	83.5	1.0	1.2	1.0	0.301	10.5	1.0	2.500	0.122	0.750	....
Dammaropsis kingiana	1	72.4	1.1	5.0	3.5	0.385	5.4	3.0	1.459	0.128	0.465	....
Gnetum gnemon	1	93.0	0.1	0.5	0.4	0.211	nil	4.0	.....	.....	.....	....
Hibiscus manihot	7	81.0	0.5	4.0	10.0	0.421	nil	0.5	.....	.....	.....	....
Lumnitzera racemosa	1	88.5	0.3	3.0	4.0	0.273	0.1	0.1	.....	.....	.....	....
Manihot dulcis	5	82.0	1.7	2.4	1.9	1.150	1.2	2.5	12.370	0.335	1.068	275
Sesbania grandiflora												
leaves	6	72.7	....	....	....	0.470	....	....	11.200	0.263	0.634	233
flowers	4	88.1	0.4	1.5	3.0	0.223	2.7	1.0	0.151	0.102	0.425	68
Tetragonia expansa	4	88.5	0.5	1.0	0.5	0.685	1.5	5.0	1.494	.....	.....	28
Wedelia biflora	2	89.0	0.5	4.0	1.0	0.483	nil	0.5	.....	.....	.....	10
(f) Saps and juices												
Nipah fruticans—sap	1	81.0	....	....	....	....	10.0	nil	.....	.....	.....	....
Cocos nucifera												
sap (fresh toddy)	4	82.0	....	....	....	....	14.0	nil	.....	.....	.....	....
water	12	95.0	0.5	....	0.3	0.093	2.5	nil	.....	nil	.....	0.3
(g) Others												
Cajanus pseudocajan (dry)	6	10.0	2.5	8.0	2.5	4.140	0.1	39.7	.....	0.006	.....	21
Dried fish	1	7.5	1.5	....	7.0	8.854	6.3	1.0	.....	.....	.....	....
"Fungus"	1	85.0	trace	0.3	3.5	0.392	3.5	0.5	.....	0.016	2.003	....
Oryza sativa												
Indian, white	4	11.0	0.2	0.2	3.6	0.771	nil	66.5	.....	0.055	1.690	....
Thailand, white	4	11.9	0.3	0.2	1.7	0.732	nil	73.0	.....	0.079	1.340	....
Fiji, white	3	11.4	0.4	0.2	2.0	0.772	nil	70.5	.....	0.075	1.570	....
Fiji, padi	5	10.6	....	....	1.7	0.014	nil	48.5	.....	0.266	3.720	....
Phaseolus lunatus	1	70.0	0.5	2.1	0.8	0.742	17.0	8.7	.....	0.222	1.293	....

TABLE 3

Amino acid composition of taro and yam[1]

Amino acid	Taro	Yam	
		White variety	Red variety
Alanine	6.7	4.3	4.1
β-Alanine	6.5	0.4	0.5
α-amino-*n*-butyric acid	5.3	0.8	1.0
Arginine	12.6	15.1	14.8
Aspartic acid	0.8	9.0	9.5
Cystine	1.7	0.6	0.5
Glutamic acid	0.6	6.1	6.8
Glycine	6.9	1.7	1.7
Histidine*	3.1	3.6	3.4
Hydroxyproline	−	+	+
Leucine*	2.9	3.5	3.4
Iso-Leucine*	0.4	2.2	2.6
Lysine*	8.4	4.2	4.0
Methionine*	3.0	0.5	0.6
Phenylalanine*	2.1	2.5	2.5
Proline	+	+	+
Serine	5.5	5.1	5.3
Threonine*	10.0	2.5	2.5
Tyrosine	1.5	1.6	1.8
Valine*	4.3	3.3	3.5
Ammonia	10.0	11.9	11.5
Total Nitrogen[2]	210 mg/100 g	268 mg/100 g	346 mg/100 g

[1] Expressed as amino nitrogen as a percent of total nitrogen.
[2] Fresh weight basis.
* Amino acids essential for man.

(a) Roots and Tubers

Taros

Colocasia antiquorum, Colocasia esculenta, Xanthosoma sagittifolium and *Cyrtosperma chamissonis* were examined. In all, some 20 different samplings of tubers drawn mainly from New Caledonia, were studied for their proximate compositions. Four samples of the leaves of *Colocasia esculenta* were analysed for proximate composition; one sample each of *Colocasia esculenta, Cyrtosperma chamissonis* and *Xanthosoma sagittifolium* tubers were examined for the vitamin content.

From the figures obtained, it can be said that all the taros are poor sources of carotene, ascorbic acid, niacin and fat, but fair sources of thiamine. The *Colocasia* and *Cyrtosperma* are better sources of protein than the *Xanthosoma*, but none of the taros constitutes a good protein source. Excepting for the *Colocasia antiquorum* and the *Cyrtosperma* the total nitrogen figures are lower than the average figures given in the literature *(17)*.

The leaves of *Colocasia esculenta*, however, constitute a reasonable source of protein, and a mixture of taro tubers and leaves would be expected to give a good balanced diet. In many areas both tubers and leaves are eaten together.

It will be seen from Table 3 that the taro has a very low content of iso-leucine, aspartic acid, and glutamic acid. This was observed with all the hydrolyzates examined. The arginine and threonine contents are high, the methionine content is extremely good for a vegetable protein, whilst the content of the other amino acids is fair to good. β-alanine was

found in nearly all samples examined in approximately the same concentration as for alanine. In all the *Colocasia* examined a strong and distinct peak was found between alanine and valine corresponding to the position of *a*-amino-*n*-butyric acid when a synthetic mixture containing this amino acid was chromatographed. Circular paper chromatograms (Giri, *8*) were run using material collected from a column and pure synthetic *a*-amino-*n*-butyric acid. Both spots had the same Rf value and, when mixed, moved as a single spot. From this evidence it was concluded that *Colocasia* contain *a*-amino-*n*-butyric acid.

An unknown peak was observed in several samples, coming just after valine, but insufficient material was obtained to enable any identifications to be made.

Sweet potatoes

All the sweet potatoes examined were *Ipomoea batatas*. Quite big differences were found in the colour and texture of the tubers, the colour varying from a dead white to a deep orange. The carotene content was found to be correlated with the depth of colour. This relationship has been the subject of work by Arthur *et al (2)*.

Eighteen samplings were examined for proximate composition, 10 "white" varieties and 8 "yellow" varieties. There was little or no difference in the proximate composition between the 2 varieties except that the total nitrogen figure of the white varieties was higher than the yellow varieties, both on a dry and fresh weight basis.

The ascorbic acid content of sweet potatoes was considerably higher than all the other roots examined, and fell within the range of published figures. The thiamine content was low but the niacin content was fair. The white variety appeared to have approximately three times as much niacin as the yellow variety, but insufficient analyses were made to know whether these figures were significant.

Yams

Two species of yams were examined—*Dioscorea alata* and *Dioscorea pentaphylla*. In each species there were two marked varieties based on colour differences. The *D. alata* varied in colour from white to a purplish red, and the *D. pentaphylla* from green to a deep purple. Two samplings of both varieties of *D. pentaphylla* were examined for proximate composition and 21 samplings of *D. alata*, 8 red varieties and 13 white varieties were examined.

The proximate composition of both varieties of *D. pentaphylla* was similar on a dry weight basis, although the total nitrogen and water content of the green variety did appear to be higher than the purple variety, but from only 2 samplings it is hard to draw any valid conclusions. The vitamin content of both varieties was low, there being only traces of ascorbic acid and very little thiamine and niacin.

For the red and white varieties of *D. alata*, the proximate composition was the same on a dry weight basis although there was a considerable difference in the water content of both varieties. The water content of the white variety ranged from 51.5–58%, with a mean value of 54.5%, and the results in Table 2 are based on this mean figure of 54.5%. The water

content of the red variety ranged from 62.0–73%, with a mean value of 68%, this last figure being used as the basis for the results.

The vitamin content of *D. alata* was also low although higher than *D. pentaphylla*. The ascorbic acid content ranged from a trace to 8 mg%. The thiamine figures were higher than those published for Hawaiian varieties *(11)*, but the niacin content was only about half the Hawaiian values. In each case it was found that the red or purple varieties had a higher carotene content than the white or green varieties. White varieties were found to have at the most only a trace of carotene and in several samples no carotene could be detected.

The red *D. alata* was not a good source of carotene containing only 0.10–0.15 mg%. The *D. pentaphylla* was a better carotene source, having 0.55–0.73 mg%. Insufficient samples were examined, however, to be able to draw any firm conclusions on the possible relation of colour to carotene content in yams.

The amino acid composition of both varieties was very similar on the basis of amino acid percentage of total nitrogen, as is shown in Table 3. The sulphur amino acid content of the yam proteins was low and, excepting for possibly serine and glutamic acid, the content of the other amino acids may be ranked as poor to fair. Proline and hydroxyproline were both present. A small amount of β-alanine was also found, together with a small amount of α-amino-n-butyric acid. This latter amino acid was also found in taro and coconut protein.

Banana corm

One sampling of *Musa paradisiaca*, var. *sapientum oleracea* was obtained. This corm comes from a fruitless variety of banana which in outward appearance resembles the ordinary banana. The corm resembles a *Xanthosoma* corm in appearance and is prepared and used in a similar manner to taro.

The main difference between this food and the taros, yams and sweet potatoes is its relatively high content of soluble sugars and consequent reduction in starch content. It contains only a trace of ascorbic acid and is low in the other vitamins.

Cassava, Manioc

Both the tubers and leaves of *Manihot dulcis* were examined. *Manihot dulcis* is the "sweet" cassava and may be eaten cooked, similarly to taro, without any intricate preparation, as the toxic glycoside content is much lower than in *Manihot esculenta* or *Manihot utillissima*, the bitter variety found in Africa, Asia, and South America.

The results obtained generally agree with other published work *(1)*. The leaves are an extremely good source of carotene, a fair source of niacin, and a good source of ascorbic acid. The tubers, however, are not a good vitamin source, having no carotene and only minor amounts of the other vitamins estimated. The tubers may generally be regarded as composed only of starch and water.

Magnana, Kudzu

Two samples of *Pueraria thunbergiana* tubers were obtained. These were the long roots of a vine and were extremely fibrous. The use of *Pueraria thunbergiana* has declined considerably and it is now mainly used only during food shortages and emergencies. It is an exceedingly poor vitamin source, but it does contain 0.2% nitrogen (total). The protein, however, was not examined. Its high fibre content detracts considerably from its food value. It is also difficult to prepare for use.

Solanum sp.

The sample of a *Solanum* tuber from Papua was received. This tuber appeared to be a good food. It contained 0.5% nitrogen on an 80% approx water basis and was a good source of carotene and a fair source of niacin.

(b) Other Starchy Foods

Breadfruit

Two samples of fresh breadfruit, *Artocarpus altilis*, were received from Western Samoa and a sample of dried breadfruit was received from the British Solomon Islands.

A note on the composition of these breadfruits has been published *(19)*. The main point of interest was the variation in the calcium content. In the dried breadfruit the calcium content was about three times higher than anticipated, and was probably due to contamination by coral dust during its preparation.

Aibon

Aibon is a starch paste preparation made from the fruit of a mangrove, *Bruguiera eriopetala*. It is used widely in parts of Netherlands New Guinea. One sample only was received and this was shown to have a relatively high protein content (about 2.5% N on a fresh weight basis). It also had a relatively high ash and fibre content.

Fern root flour

Two samples of a flour prepared from the root of *Pteris moluccana* were received from Netherlands New Guinea. This flour is prepared by grinding the fern root and extracting the ground material with water and squeezing through "cloth." The extract is then dried in the sun. The "flour" had an extremely high fibre content, 10%, but also contained about 12% crude protein (N x 6.25).

Sago

Samples of raw sago *(Metroxylon sagu)* and of 6 sago preparations were received from Netherlands New Guinea. Method of preparing the various sagos is as follows:

Raw Sago: The trunk of the sago palm is split and the pith is removed by grating or crushing with a stone adze or sharpened piece of bamboo. The crushed sago is kneaded by hand whilst it is being washed, and the starch and water mixture is collected and filtered through a fibrous "cloth." Then the drained starch is partially dried and either stored in small palm leaf basket or processed further.

Sago Boengkoes: The raw sago is made into a thick paste or dough is drawn out into a rod shape, wrapped in a palm leaf and roasted on a fire.

Sago bulu: Hollow bamboos are filled with a raw sago paste and cooked over a fire.

Sago ega: The sago is pressed into brickettes and wrapped in sago palm leaves.

Sago lempeng: The raw sago is placed in tins or other receptacles and baked until it is hard and dry.

Sago senole: The raw sago is mixed with grated coconut and baked in the form of a flat cake or biscuit.

Sago sep: This is similar to sago lempeng except that the raw sago is baked into thin flat biscuits on a hot stone or other flat surface.

Excepting for sago senole it can be said that all sago preparations examined consisted only of starch and water in varying proportions with small amounts of extraneous ash and fibre derived during preparation. Raw sago contained a small quantity, 35 mg, of total nitrogen, but this seems to be lost during the various preparative procedures.

In all, 4 different shipments of sago preparations were received, each containing at least 2 samplings of the sago preparation. As would be expected, the water content varied considerably and average values have been taken. The sago ega samples received, however, fell into 2 general classes on a water content basis and the results of these 2 classes are given.

From a nutritional point of view, the only preparation which had any value, other than as a source of calories, was sago senole. The addition of grated coconut added nitrogen and fat, both of which are either completely lacking, or present only in trace proportions in the other sago preparations examined.

Polynesian arrowroot, Tacca

One sample of tacca flour *(Tacca leontopetaloides)* was examined. This flour was prepared from tacca tubers in the Tuamotu Islands by washing the grated tuber twice with sea water, once with fresh water, and then drying in the sun. This procedure removed a bitter substance which was present in the fresh tubers.

The flour consisted essentially of starch and water, but did contain 3% ether extractable material.

(c) Fruits

Pawpaw (Papaya)

Eight samplings of the ripe, fresh fruit of *Carica papaya*, 5 samplings of the green fruit, and 5 samplings of young leaves were examined for proximate composition.

Both green and ripe fruit had a very high water content. On a dry weight basis, the total nitrogen values were good. The leaves had quite a high protein content. The results obtained for leaves and fruit agreed reasonably well with figures that had been previously published *(17)*.

Eugenia fruit

Two samples of the fruit of *Eugenia jambos* were examined for proximate composition. As a food they may be regarded as being just average, not possessing any outstanding properties.

Fig

A sampling of a New Caledonian native fig, *Ficus racemigera,* was examined. The fruit was small, about the size of an index fingernail in diameter, but sweet.

The carotene content was good, nearly 1 mg%, but the content of other vitamins was low.

Pandanus

The three samplings of fresh pandanus fruits *(Pandanus sp.)* were received from Tarawa and two prepared samplings from Papua. Two samplings of the roasted nuts of *Pandanus julianetta* were also received from Papua. The pandanus constitutes an important part of the diet of the atoll peoples of Micronesia and Polynesia.

The pandanus from Tarawa were a deep orange in colour. Miller *et al (12),* stated the yellow orange colour was due to carotenoid pigments. However, it was found that the pigments in the Tarawa pandanus was water soluble and gave typical anthocyanin reactions. The carotene content was found to be only 0.011 mg% (range 0.009–0.013 mg%), which is much below Miller's figures, 0.184–1.242 mg%. Unfortu tely, the New Guinea pandanus samplings had not been prepared for carotene assays. The niacin content of the New Guinea pandanus was good. It was found that the Tarawa pandanus contained an extremely active ascorbic acid oxidase. Tests were made on New Caledonian pandanus, which are inedible, but these were found to possess no ascorbic acid oxidase activity.

Pandanus "keys" are extremely fibrous, and the analyses were made on edible portions of the Tarawa pandanus omitting much of the fibre which would normally be discarded after chewing. This would account for the big difference in fibre content between the Tarawa and New Guinea pandanus, as the latter were prepared by chopping up the whole "edible" segments. The total nitrogen content of both species of pandanus was good. The New Guinea pandanus also contained a high ether extractable content.

The *Pandanus julianetti* nuts were prepared by roasting the pandanus keys and then removing the edible nuts. These nuts had a high niacin and a good crude protein content. The amount of ether extractable material was also high. The high fibre content was caused by cellulose strands which adhere closely to the nut, however, much of this cellulose is discarded after chewing.

(d) Nuts

Coconut

Although the coconut, *Cocos nucifera,* has been the subject of much work previously, many determinations were made on the nut at various stages of ripening, and for specific constituents. Values obtained for the meat and water of the nut agree generally with figures which had been published previously *(16).*

The coconut "apple," a spongy mass which develops inside the coconut when the nut is sprouting, was of interest, as on some atolls this is often the only fresh "vegetable" available, and is widely eaten both raw and cooked. The water content was high, but on a dry weight basis it had a high content of protein and soluble sugars.

"Toddy," the sap of the coconut palm is very widely used as a drink It is prepared by binding a flower spathe just before it opens and slicing off the end. The sap oozes from the cut end and is allowed to drip into a container and is collected once or twice a day. After each collection the end of the spathe is shaved to expose a fresh surface. The process continues until the spathe has been shaved away completely. If allowed to stand, the toddy rapidly ferments. In some areas only unfermented toddy is drunk, in others only fermented toddy, and in still others both forms are consumed.

Polynesian chestnut

Three samplings of *Inocarpus edulis* were obtained, two from Fiji and one from Western Samoa. These nuts had a low fat and a high starch content. The protein content was comparable with that of the coconut. Similarly to the coconut, the ascorbic acid content was low but, by contrast, the thiamine content was high.

(e) Leaves

Bamboo shoots

One sample of bamboo shoots, *Bambusa sp.*, was received from Netherlands New Guinea. These had a relatively high protein content, but otherwise were of little interest.

Pumpkin leaves

One sample of the leaves of *Cucurbita pepo* was received from Papua. This was found to be a good source of carotene and protein, and was rich in soluble reducing sugars.

Dammaropsis kingiana leaves

The one sample of *Dammaropsis kingiana* leaves received from Papua was found to be a good source of carotene and protein.

Gnemon leaves

One sample of the leaves of *Gnetum gnemon* from Netherlands New Guinea was examined. This sample showed a very high water content, and unlike the other leaves examined, a high starch content, about 60% on a dry weight basis.

Hibiscus leaves

Leaves of *Hibiscus manihot* are widely eaten as a green vegetable and several samplings were obtained in New Caledonia. These showed a good protein content and a high content of ether extractable matter. The water and fibre contents were also high.

Mangrove leaves

Leaves of the mangrove, *Lumnitzera racemosa,* are used by the natives of New Caledonia as an emergency food during periods of food shortage. One sample was examined which showed a high fibre and ether extract content and a reasonable protein content.

Sesban leaves and flowers

Sesbania grandiflora is a large tree found throughout Indonesia and the Pacific. In many areas it is an ornamental tree, but it is used in Indonesia,

Netherlands New Guinea and French Oceania as a food tree; both the leaves and flowers being eaten.

The leaves were found to be an extremely rich source of carotene and a very good source of ascorbic acid and thiamine. The protein content was also found to be good. On the other hand, the flowers were only a fair source of vitamins and protein.

New Zealand spinach

New Zealand spinach, *Tetragonia expansa,* is found throughout the Pacific. On the low atolls, it is one of the few green vegetables. It grows generally along beaches and in sandy regions. Samples were obtained from New Caledonian beaches.

Wedelia biflora

Widelia biflora is a plant with succulent leaves which are used as a cooked green vegetable by the natives in New Caledonia. Two samples were examined.

(f) Saps

Nipah sap

A "toddy" is prepared from the palm, *Nipah fruticans,* similarly to the coconut "toddy." One sample of unfermented Nipah "toddy" from Netherlands New Guinea was examined for its sugar content.

(g) Others

Pigeon pea

A type of pigeon pea, *Cajanus pseudocajan,* which is eaten both fresh and dry was examined for its proximate composition. The dry pulse was found to have a high protein and starch content and would constitute a good food, even though the fibre content tended to be high. The thiamine content was very low.

Dried fish

In the British Solomon Islands, fish flakes were prepared by cleaning the fish and boiling them for 30 min in a mixture of 3 parts sea water and 1 part coconut water. After cooking, the bones were removed and the flesh was broken into small pieces which were slowly dried for 24 hr over a small fire. The final product consisted of yellow-brown flakes ¼–½ inch long, which had a pleasant though slightly smoky taste.

These flakes had a high protein content, were very palatable, and kept well *(20)*.

Fungus

A sample of fungus from Papua was examined. This was found to be a good source of niacin and a fair source of protein.

Rice

Samples of rice were received from Fiji. These samples were of imported white rices from India and Thailand and a white rice grown and milled in Fiji, and a Fijian padi. The results generally agree with the published figures.

Bean

A sample of *Phaseolus lunatus* was received from Papua. This was a good source of niacin and protein and contained a high content of soluble reducing sugars.

CONCLUSIONS

From the analytical results obtained, it would seem that an adequate and balanced diet could be derived from the local foodstuffs. However, it must be realized that the foods examined were drawn from many parts of the Pacific and therefore they would not all be available in any one area. In particular, is this true of those people existing on an extremely limited diet, such as the sago eaters of New Guinea. The analytical figures obtained for the various sago preparations and observed intakes of other foods reported by Oomen and Malcolm *(15)* would indicate that these people are grossly lacking in such essential food constituents as protein. However, there are many other factors that would have to be investigated before any firm conclusions could be made.

From the amino acid analyses, the quality of the proteins of taro appear to be good, whilst those of yam are poor. It would be interesting to see whether there is any correlation between the colour of sweet potatoes and their nitrogen content. On the figures presented it would seem that there could be. The need for a greater knowledge of the amino acid composition of South Pacific foods is clearly indicated.

A study which should be undertaken, and which was begun in this work, is the composition of the coconut at all stages of maturity from the "drinking nut" stage to the sprouted nut stage. Time did not allow the study of the amino acid composition of coconut water at various growth stages to be made. A definite change in the sugars and the total nitrogen of the water during maturation of the coconut was observed and it would be interesting to see if there was any change in the free amino acid content and the relation of the various amino acids with each other. At present it can be said that the coconut is an extremely valuable food which should be more thoroughly exploited as such. The present tendency is for the food role of the coconut to be superseded by its "cash crop" role. In many areas it is being replaced as a food by cassava and similar foods, a tendency which is to be deplored.

The results reported for pandanus are at considerable variance with those of Miller *et al (12)*, especially regarding the carotene content. No previous reference can be found describing the nature of the pigments present in the fruit. It appears to have been generally accepted that the colour was due to carotenoid pigments and that pandanus was a good source of carotene. It would appear from the results reported here that this is not the case with all pandanus, and in fact the pandanus from the Gilbert Islands are a poor source of carotene. Unfortunately, the pandanus growing in New Caledonia are of inedible species, and do not appear to colour to the same extent as those present in other parts of the Pacific. Further work on this subject could not be undertaken because of the difficulty in obtaining suitable material.

Several of the lesser known foods examined would make valuable additions to the diets of many groups in the South Pacific region if they were

introduced. The nuts of *Pandanus julienetti* are a good source of protein, fat, and niacin. *Sesbania grandiflora* leaves are an extremely rich source of carotene and ascorbic acid and a fair protein source. Pawpaw leaves are a good protein source and have been reported in the literature as being a rich source of carotene. The pigeon pea is a good protein source.

The dried fish and smoked bread fruit, both from the British Solomon Islands, are good examples of local practices in food preservation, which could be easily adapted to other parts of Pacific and which could become valuable supplementary foods or items of trade.

The exposure of the indigenous peoples of the South Pacific to European influences over the last century or century and a half has had a great effect on the peoples themselves and on the economy of the islands which they inhabit. Some of the peoples, especially in parts of New Guinea, still live in Stone Age communities, and know only a strict subsistence economy, whilst others, such as the peoples of New Caledonia and parts of the French Establishments in Oceania, have been more influenced by Europeans and Chinese and have neglected their traditional subsistence crops such as taro, yams and coconuts for cash crops such as coffee and copra. A pattern is now developing whereby the economy of the South Pacific islands is being based more and more upon the production of some staple foods supplemented by imported foods purchased from the proceeds of the sale of cash crops.

In some areas, Europeans and Asians have introduced a number of good food plants, such as various types of beans. This has been counterbalanced, however, by the introduction of European cooking utensils and methods which are often inferior to the traditional native methods in preserving the bulk of the nutrients.

That there should be a change-over from a subsistence economy to a cash economy and from traditional practices to European methods of food preparation is to be expected. It should be the responsibility of territorial administrations to assist in this transition and every attempt should be made to maintain adequate nutritional standards, and to improve existing standards where these are not as high as they could be. Two points which should be borne in mind are:

(a) every effort should be made to improve certain species and varieties of the traditional food plants and to keep these in the new agricultural patterns;

(b) the island peoples should be educated in the relative values of local and imported foods and in the various methods of food preparation.

The substitution of cassava and foods of this nature for taros is a retrogressive step. These foods lead to a marked imbalance in the diet and a general falling off in the health of the peoples.

SUMMARY

Results are presented for the proximate composition of 62 foods, for the amino acid composition of two staple foods and for the vitamin content of 38 foods used in the South Pacific.

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VARIATIONS IN CHEMICAL COMPOSITION OF DIFFERENT PARTS OF HALIBUT FLESH

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There is great variation in the chemical composition of fish flesh. Data included in previous reports *(6, 7)* indicate that variations in composition of the fish depend on a number of factors including (a) size, (b) sexual maturity, (c) season and area of capture, (d) method of storage, and (e) part of fish from which sample is taken.

Few data on the composition of halibut are found in the literature. Watt and Merrill *(8)*, in their comprehensive *Composition of Foods* make one reference to halibut, listing it as having 18.6% protein and 5.2% oil. These values have been quoted frequently *(4)*. Bailey *(2, 3)* found that the oil content in a series of small specimens ranged from 0.15% to 5.79%. In more recent work, McBride and MacLeod *(5)* found small- and medium-size halibut to contain 58.9 and 62.0 mg/100 g, respectively, of sodium and 452 and 475 mg/100 g, respectively, of potassium. The average composition of 11 small halibut analyzed in our laboratory in 1938 was as follows: moisture 78.8%, oil 1.82%, protein 18.6%, and ash 1.20%. Seven medium-size halibut analyzed here in 1953 showed about the same average composition: moisture 78.0%, oil 2.70%, protein 19.1%, and ash 1.20%.

Because no data were available on the composition of large fish or on the composition of different parts of the same fish, an intensive study was begun to determine variations in chemical composition of halibut flesh and to correlate the data with (a) size of the fish, (b) part of the fish from which sample was taken, and (c) manner in which the fish was refrigerated on board the vessel.

Since the halibut used in this work were both large in size and highly valuable, a study of the present scope would have been financially impossible except that, fortunately, most of the halibut were made available free as the result of a scientific exploratory fishing project in progress at the time the study was begun.

The data obtained indicate that the edible portion of this important Pacific Northwest fishery product is much higher in protein and much lower in oil than was previously thought and that it has a lower sodium content than have most salt-water fish and some fresh-water fish.

EXPERIMENTAL

Collection of specimens. Eight iced and 37 frozen specimens of halibut ranging in size from 2 to 95 pounds were collected during the summers of 1957 and 1958. They were captured in the area that extends from the north end of Vancouver Island to Kodiak Island. The 8 iced halibut were purchased in Seattle and taken directly from fishing vessels. These iced halibut had been eviscerated on capture, then stored in ice for not more than 7 to 10 days, and were in good condition. The 37 frozen halibut were obtained from the U. S. Fish and Wildlife Service's charter vessel *Tordenskjold* in 1957

and from its exploratory fishing vessel *John N. Cobb* in 1958. These halibut, frozen whole immediately on capture, were kept frozen until samples were prepared.

Limitations of study. The quantity of fish included in this study was not adequate for a comparison of the variations in composition with area and season of capture. Since the iced fish had been eviscerated prior to delivery and since the viscera of the frozen fish were usually not thawed, no attempt was made to classify the halibut according to sex.

Definition of terms. Halibut is a flat fish with both eyes on the same (upper) side. It swims on its side, with reference to head and viscera. The upper side is dark green and is called the right side. The lower side is white and is called the left side. Commercial cuttings are either longitudinal fillets or cross-sectional steaks. On either side of the head is a small pocket of flesh called the cheek. Underneath the skin along the center of each side is a thin layer of dark meat. The soft layer of flesh next to the viscera is called the belly flesh. Evisceration of the fish forms 2 thin flaps of flesh called belly flaps. The carcass contains a spine with 2 sets of rib-like bones that grow at right angles to each other. The 4 quarters of flesh thus formed are called fletches. The nape of a fish is the section of flesh next to the head.

Preparation of samples. Samples were from 10 subcommercial-size frozen halibut (under 5 lb), 19 small-size frozen halibut (5 to 20 lb), and 8 large-size frozen halibut (20 to 95 lb) and from 8 large-size iced halibut (20 to 95 lb). Thus, there were samples from 37 frozen and from 8 iced fish.

The frozen halibut were partially thawed, and samples removed while the flesh was cold or partially frozen. Before the samples were taken, each fish was washed and then measured for length and weight. Using factors of 10% for the heads and 12% for the viscera, we calculated what the weights would be with heads and viscera removed as in commercial practice.

Samples from the 10 subcommercial-size frozen fish were prepared from the combined right and left fillets only. Samples from the 19 small-size frozen fish were from the combined right and left cheeks, dark meat, belly flaps, and fletch tips and from steak sections from the right and left sides of nape, center, and tail parts. Samples from the 8 large-size frozen fish were taken in the same manner as were the samples from the 19 small-size frozen fish except that 2 additional steak samples from the front of center and rear of center were taken. All parts were from separate sides.

Samples from the 8 large-size iced fish were taken in the same manner as were samples from the 8 large-size frozen fish. Samples from the iced fish were taken while the fish were still chilled.

Each sample, whether from frozen or iced halibut, was ground in a meat cutter, vacuumed sealed in a ½-lb can, and stored at −28° C until analyzed.

Methods of analysis. Each sample was analyzed for moisture, oil, protein, and ash contents and for sodium and potassium contents by standard methods of analysis *(1)*.

RESULTS AND DISCUSSION

Fillets of the 10 subcommercial-size frozen halibut had a fairly uniform composition. Average composition was: moisture 79.4%, oil 0.6%, protein 19.8%, ash 1.35%, sodium 45 mg/100 g, and potassium 430 mg/100 g.

Composition data for the 35 halibut (27 frozen and 8 iced) of commercial size are shown in Tables 1, 2, and 3. The fishing industry classifies halibut into 6 weight groups ranging from 5 to over 80 lb. The small number of specimens in each of the larger groups in the present study made such a classification impractical. For comparative purposes, the 3 weight groups used in the tables are small (5 to 20 lb), large (20 to 95 lb), and combined small and large (5 to 95 lb). The last classification—combined small and large—is included to permit a comparison of values for the total weight range of commercial specimens.

Variations in composition of the different parts of the 27 frozen fish with size and part of fish when sides of fish were combined are shown in Table 1. The cheeks were low in oil, protein, ash, and potassium, and very high

TABLE 1

Variations in composition of frozen halibut flesh with size and part of fish when sides of fish are combined

Part of fish	Moisture		Oil		Protein		Ash		Sodium		Potassium	
	Av	Range	Av	Range	Av	Range	Av	Range	Av	Range	Av	Range
	%	%	%	%	%	%	%	%	*Mg/100 g*	*Mg/100 g*	*Mg/100 g*	*Mg/100 g*
Group A (19 fish) small—5 to 20 lb												
Cheeks	79.5	78.2–81.0	0.7	0.6– 0.9	19.4	18.1–20.4	1.26	1.14–1.37	112	84–166	351	288–387
Dark meat	75.2	67.9–78.5	5.7	2.2–13.2	18.2	16.6–19.4	1.33	1.12–1.69	82	58–120	368	318–395
Belly flaps	78.4	75.9–81.8	1.0	0.5– 2.6	20.2	17.5–21.3	1.32	1.19–1.50	91	72–115	379	319–409
Fletch tips	78.0	76.2–81.7	0.8	0.5– 1.2	20.9	17.7–22.0	1.36	1.20–1.45	59	49– 63	438	391–461
Nape	78.3	76.8–81.3	0.7	0.4– 0.9	20.9	18.2–22.5	1.40	1.28–1.48	47	37– 76	458	405–496
Center	78.0	76.5–81.2	0.6	0.4– 0.8	21.0	18.1–22.7	1.40	1.28–1.53	40	32– 55	466	403–504
Tail	78.0	76.1–81.0	0.6	0.4– 0.8	21.3	19.6–22.8	1.38	1.23–1.46	53	44– 74	444	382–479
Combined (N.C.T.)	78.1	76.1–81.3	0.7	0.4– 0.9	21.1	18.1–22.8	1.39	1.23–1.53	47	32– 76	456	382–504
Group B (8 fish) large—20 to 95 lb												
Cheeks	79.5	78.4–80.3	0.7	0.4– 1.4	19.4	19.1–19.7	1.25	1.22–1.31	92	74–140	388	341–424
Dark meat	72.1	66.8–77.4	10.0	4.2–16.5	17.0	15.7–17.7	1.23	1.11–1.42	81	59– 98	375	364–394
Belly flaps	77.0	75.2–78.1	2.1	0.8– 2.8	20.7	19.5–22.2	1.30	1.21–1.36	84	68–108	376	350–416
Fletch tips	74.3	69.1–78.0	6.4	0.9–14.0	18.6	16.4–21.1	1.19	1.05–1.36	84	60–120	347	271–449
Nape	77.3	76.5–77.9	0.9	0.6– 1.5	21.3	20.1–22.5	1.38	1.33–1.45	51	45– 60	443	412–475
Center	77.3	76.2–78.2	0.7	0.3– 1.1	21.9	21.2–23.2	1.41	1.34–1.45	36	30– 45	465	415–508
Tail	77.5	76.4–79.3	0.7	0.5– 1.1	21.1	20.0–22.0	1.34	1.22–1.39	86	53–115	372	348–470
Combined (N.C.T.)	77.4	76.2–79.3	0.8	0.3– 1.5	21.4	20.0–23.2	1.38	1.22–1.45	58	30–115	427	348–508
Group C (27 fish) sm & lge—5 to 95 lb												
Cheeks	79.5	78.2–81.0	0.7	0.4– 1.4	19.3	18.1–20.4	1.26	1.14–1.37	106	74–166	362	288–404
Dark meat	74.3	66.8–78.5	7.0	2.2–16.5	17.8	15.7–19.4	1.30	1.11–1.69	82	58–120	361	318–395
Belly flaps	78.0	75.2–81.8	1.3	0.5– 2.8	20.3	17.5–22.2	1.31	1.19–1.50	89	68–115	378	319–416
Fletch tips	76.7	69.1–81.7	2.9	0.5–14.0	20.0	16.4–22.0	1.29	1.05–1.45	68	49–120	405	271–461
Nape	78.0	76.5–81.3	0.7	0.4– 1.5	21.0	18.2–22.5	1.39	1.28–1.48	48	37– 76	454	405–496
Center	77.8	76.2–81.2	0.6	0.3– 1.1	21.3	18.1–23.2	1.40	1.28–1.53	39	30– 55	466	403–508
Tail	77.9	76.1–81.0	0.8	0.4– 1.0	21.2	19.6–22.8	1.37	1.22–1.46	63	44–115	425	348–479
Combined (N.C.T.)	77.9	76.1–81.3	0.7	0.3– 1.5	21.2	18.1–23.2	1.39	1.22–1.53	50	30–115	448	348–508

Note: (N.C.T.) refers to nape, center, tail.

TABLE 2

Variations in composition of frozen halibut flesh with size and part of fish when sides of fish are considered separately

Part of fish	Moisture		Oil		Protein		Ash		Sodium		Potassium	
	Av	Range	Av	Range	Av	Range	Av	Range	Av	Range	Av	Range
Group A (19 fish) small—5 to 20 lb	%	%	%	%	%	%	%	%	*Mg/100 g*	*Mg/100 g*	*Mg/100 g*	*Mg/100 g*
Right side												
Nape	78.3	76.8–81.2	0.7	0.4– 0.9	20.7	18.2–22.4	1.39	1.28–1.51	48	38– 86	458	399–508
Center	78.2	76.6–81.2	0.6	0.4– 0.8	20.9	18.0–22.4	1.43	1.28–1.56	39	31– 56	471	409–515
Tail	78.1	76.0–81.3	0.6	0.4– 0.8	21.0	18.1–22.6	1.37	1.21–1.50	50	39– 70	450	382–493
Combined (N.C.T.)	78.2	76.0–81.3	0.6	0.4– 0.8	20.9	18.0–22.6	1.40	1.21–1.56	46	31– 86	460	382–515
Left side												
Nape	78.1	76.4–81.4	0.6	0.4– 1.0	21.0	18.2–22.9	1.40	1.27–1.48	47	35– 62	460	410–497
Center	77.9	76.3–81.2	0.5	0.4– 0.7	21.2	18.2–22.4	1.43	1.28–1.54	40	30– 55	465	396–514
Tail	77.8	76.1–80.6	0.6	0.4– 0.7	21.3	18.4–22.8	1.38	1.25–1.47	55	45– 67	442	396–469
Combined (N.C.T.)	77.9	76.3–81.4	0.6	0.4– 1.0	21.2	18.2–22.9	1.40	1.25–1.54	47	30– 67	455	396–514
Group B (8 fish) large—20 to 95 lb												
Right side												
Nape	77.6	76.7–78.0	1.0	0.7– 1.9	21.3	20.8–22.1	1.37	1.34–1.44	53	45– 59	440	412–484
Center	78.0	76.3–80.0	0.7	0.3– 1.1	21.7	19.9–23.4	1.37	1.29–1.44	34	29– 42	461	417–481
Tail	77.7	76.4–79.3	0.7	0.6– 0.9	20.7	19.9–21.9	1.31	1.19–1.37	89	60–112	367	321–408
Combined (N.C.T.)	77.8	76.3–80.0	0.8	0.3– 1.9	21.2	19.9–23.4	1.35	1.19–1.44	59	29–112	423	321–484
Left side												
Nape	77.1	76.1–77.9	0.9	0.6– 1.3	21.4	20.8–22.4	1.37	1.31–1.45	49	41– 60	433	402–466
Center	77.3	75.8–79.2	0.6	0.3– 1.1	22.2	21.5–23.2	1.44	1.39–1.55	38	33– 48	442	413–493
Tail	77.6	76.1–79.3	0.8	0.5– 1.0	21.2	20.3–22.3	1.33	1.26–1.42	86	45–118	356	319–382
Combined (N.C.T.)	77.3	75.8–79.3	0.8	0.3– 1.3	21.6	20.3–23.2	1.39	1.26–1.55	58	33–118	410	319–493

TABLE 2 (continued)

Part of fish	Moisture		Oil		Protein		Ash		Sodium		Potassium	
	Av	Range	Av	Range	Av	Range	Av	Range	Av	Range	Av	Range
Group C (27 fish) sm & lge—5 to 95 lb	%	%	%	%	%	%	%	%	*Mg/100 g*	*Mg/100 g*	*Mg/100 g*	*Mg/100 g*
Right side												
Nape	78.1	76.7–81.2	1.0	0.4– 1.9	20.9	18.2–22.4	1.39	1.28–1.51	49	38– 86	453	399–508
Center	78.2	76.3–81.2	0.6	0.3– 1.1	21.1	18.0–23.4	1.41	1.28–1.56	38	29– 56	469	409–515
Tail	78.0	76.0–81.3	0.6	0.4– 0.9	20.9	18.1–22.6	1.36	1.19–1.50	60	39–112	428	321–493
Combined (N.C.T.)	78.1	76.0–81.3	0.7	0.3– 1.9	21.0	18.0–23.4	1.39	1.19–1.56	49	29–112	450	399–515
Left side												
Nape	77.9	76.1–81.4	0.7	0.4– 1.3	21.1	18.2–22.9	1.39	1.27–1.48	47	35– 62	453	402–497
Center	77.7	75.8–81.2	0.6	0.3– 1.1	21.5	18.2–23.2	1.43	1.28–1.54	39	30– 55	459	396–514
Tail	77.7	76.1–80.6	0.6	0.4– 1.0	21.3	18.4–22.8	1.37	1.25–1.47	63	45–118	420	319–469
Combined (N.C.T.)	77.8	75.8–81.4	0.6	0.3– 1.3	21.3	18.2–23.2	1.40	1.25–1.54	50	30–118	444	319–514
Group D (8 fish) large—20 to 95 lb												
Right side												
Cheek	79.3	77.8–79.8	0.8	0.4– 2.1	19.4	18.9–19.8	1.27	1.24–1.31	95	69–141	357	334–392
Dark meat	71.7	66.8–76.9	10.9	4.8–16.8	16.7	15.3–17.4	1.24	1.05–1.58	81	77– 87	303	265–347
Belly flap	76.9	75.7–77.9	1.9	0.8– 2.9	20.7	19.3–22.1	1.33	1.23–1.39	87	66–108	385	318–425
Fletch tip	75.0	71.8–77.9	5.3	0.5–10.0	19.3	17.3–22.1	1.22	1.10–1.41	88	49–119	374	313–467
Left side												
Cheek	79.5	78.9–80.0	0.6	0.4– 0.7	19.4	18.9–19.8	1.22	1.19–1.25	98	79–138	336	305–361
Dark meat	73.0	76.7–77.8	8.6	3.2–16.2	17.3	15.7–18.0	1.20	1.08–1.28	88	77–109	326	279–351
Belly flap	76.9	74.7–78.6	2.2	1.1– 3.9	20.6	19.6–22.0	1.28	1.20–1.34	88	63–110	365	325–407
Fletch tip	72.3	66.3–78.1	10.8	1.2–18.0	17.0	15.1–20.6	1.11	0.97–1.31	92	61–130	298	231–432

TABLE 3

Variations in composition of halibut flesh with size and part of fish for frozen and iced specimens when sides of fish are combined

Part of fish	Moisture		Oil		Protein		Ash		Sodium		Potassium	
	Av	Range	Av	Range	Av	Range	Av	Range	Av	Range	Av	Range
	%	%	%	%	%	%	%	%	*Mg/100 g*	*Mg/100 g*	*Mg/100 g*	*Mg/100 g*
Group A (8 fish) frozen—20 to 95 lb												
Cheeks	79.5	78.4–80.3	0.7	0.5– 1.4	19.4	19.1–19.7	1.26	1.22–1.31	92	74–140	388	341–424
Dark meat	72.0	66.8–76.0	10.0	4.2–16.5	16.9	15.5–17.7	1.23	1.11–1.42	81	59– 98	375	364–394
Belly flap	77.0	75.2–78.1	2.1	0.8– 2.8	20.8	19.5–22.2	1.30	1.21–1.36	84	68–108	376	350–416
Fletch tip	74.0	69.1–78.0	7.2	0.9–14.0	18.6	15.1–21.1	1.17	1.00–1.36	86	55–130	374	292–449
Nape	77.3	76.5–77.9	0.9	0.7– 1.5	21.4	20.9–22.5	1.38	1.33–1.45	51	45– 60	443	412–475
Center	77.3	76.3–79.2	0.7	0.3– 1.1	22.0	21.2–23.2	1.41	1.34–1.45	36	31– 45	465	415–508
Tail	77.6	76.4–79.3	0.7	0.5– 1.0	21.0	19.8–21.9	1.32	1.22–1.36	86	53–115	379	350–470
Combined (N.C.T.)	77.4	76.3–79.2	0.8	0.3– 1.5	21.5	19.8–23.2	1.37	1.22–1.45	58	31–115	429	350–508
Group B (8 fish) iced—20 to 95 lb												
Cheeks	80.7	79.9–82.1	0.8	0.5– 1.1	18.5	17.0–19.1	1.10	0.98–1.26	77	65–130	354	324–398
Dark meat	67.2	51.4–78.5	16.7	3.0–37.4	15.4	11.3–18.4	1.03	0.81–1.23	56	42– 79	335	232–409
Belly flap	77.8	72.5–80.6	4.7	1.5–10.5	18.3	16.9–20.7	0.99	0.89–1.28	59	50– 64	318	278–391
Fletch tip	77.6	76.9–78.1	2.9	1.2– 5.5	19.5	16.4–20.0	1.20	1.17–1.24	48	41– 55	373	357–396
Nape	77.8	77.3–79.0	1.5	0.7– 2.3	20.6	19.7–21.9	1.25	1.16–1.35	47	37– 57	392	312–440
Center	77.8	77.0–78.8	1.2	0.7– 2.7	21.0	19.9–21.9	1.30	1.23–1.35	39	32– 46	411	377–454
Tail	78.2	77.6–79.2	1.0	0.7– 1.4	20.3	18.6–21.7	1.20	1.08–1.28	65	46– 98	350	267–399
Combined (N.C.T.)	77.9	77.0–79.2	1.2	0.7– 2.7	20.6	18.6–21.9	1.25	1.08–1.35	51	32– 98	384	267–454

Note: The fish in Groups A and B had about the same weight distribution.

in sodium. With increasing size of fish, the constituents remained about the same except that sodium decreased and potassium increased. Although the cheeks are considered to be a great delicacy by many fish gourmets, they were lower in food value than most of the other parts. The dark meat of the fish showed the greatest amount of variation. Oil content was higher in the dark meat than in any other part (up to 16%), and increased with size of fish. It averaged 5 times higher than in the belly flaps. The dark meat was low in both protein, with the minimum being 15%, and ash, with the minimum being 1.1%, and the values decreased with increasing size of fish. However, sodium and potassium contents remained about the same, as the size of fish increased. Belly flaps and fletch tips are minor parts in quantity, but of interest because of their high oil content. In both parts, the oil content increased with size of fish, but was 2 to 3 times higher in tips than in belly flaps. Maximum values were 3% for belly flaps, 14% for fletch tips. Steak sections, which represent the major part of food servings, showed the most uniform composition and the least variation with size of halibut. Since the front of center and the rear of center sections had intermediate values between the adjacent nape, center, and tail sections, only these latter three are included in the table. Oil content was usually less than 1%, the average being 0.7%. Highest values were found in the nape. The center section was usually higher in protein and potassium and lower in sodium than either the nape or the tail. The high sodium content of some tail sections was surprising, since it was sometimes 2 to 5 times as high as in the center part.

Variations in composition of frozen halibut flesh with size and part of fish showed little variation between right and left sides (Table 2) except in oil content. Only in the larger fish were analyses made on the sides separately for cheeks, dark meat, belly flaps, and fletch tips. In these parts, the oil varied from 0.4% to 16.8%. In the dark meat, the oil was often twice as high in one side as in the other, but neither side predominated consistently. Fletch tips were usually higher in oil content than were belly flaps, and in both parts, higher values were found more frequently in the left or lower side. In a number of the larger fish, all 4 fletch tips were analyzed, and in these, the dorsal tip consistently was higher in oil than was the ventral. Steak sections were uniformly low in oil, seldom exceeding 1%, and little difference was found between the 2 sides. The other constituents were fairly uniform in the 2 sides, except where variations in oil content caused small fluctuations.

Variations in composition of the flesh with size and part of fish for both frozen and iced specimens, when sides of fish are combined, are shown in Table 3. As might be expected, iced fish were slightly lower in protein, ash, sodium, and potassium than frozen specimens. The decrease represented drops of 5% for protein and about 10% for the other constituents in comparison to the amounts of these constituents in the frozen fish. Most halibut are brought to port in ice; hence these slightly lower values represent the amounts normally found in halibut flesh. Iced fish were much higher in oil than were frozen specimens, but this higher oil content cannot be attributed to storage method. In iced halibut as in frozen, the dark meat had the highest amount of oil, with a maximum of 37.4%. The right side

was considerably higher in oil than was the left for both dark meat and belly flaps. In one sample, values were 40.5 and 34.3, which gave the high average of 37.4% quoted above.

The amount of moisture in halibut flesh and its variations have not been emphasized in the above discussion, since moisture usually varies inversely with oil content. For steak sections, the moisture averaged about 78%, which is in the normal range for most fish.

The small amount of flesh between the skin and fin, external to the flesh of the fletch appeared to be so oily in nature that, to satisfy our curiosity, we took samples from a few of the larger fish. These samples had an oil content of 20 to 40% and averaged about 30%. The oil was light in color, very fluid, and had but slight odor. This part of the halibut is never included with the edible portions removed from the fish, but it is interesting that the small amount of flesh found in this exposed part of the fish was composed of about one-third oil. This oil appeared to be of high quality.

SUMMARY

Chemical analyses of the various parts of halibut flesh taken from frozen and iced specimens collected in the summers of 1957 and 1958 from Vancouver Island to Kodiak Island showed great variation in composition. Major variations were found in the minor parts such as dark meat, belly flaps, and fletch tips. Differences between right and left sides were not significant except for the oil content. Steak sections were quite uniform in composition and averaged less than 1% oil, about 21% protein, and about 50 mg of sodium per 100 g of fish. Iced halibut were considerably lower in ash and in sodium and potassium than were the frozen. Practically all halibut flesh is frozen. Since it is not usually held in refrigerated brine on board the vessel and ordinarily not brine dipped in the freezing process, it provides a source of high protein that can be included even in diets most restricted in sodium.

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CYSTINE AND TOTAL SULFHYDRYL CONTENT OF UNSPOILED AND SPOILED SHRIMP

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Chemical indices to determine the extent of spoilage of fish primarily have been used in attempts to correlate the degree of spoilage with the amounts of intermediate and end products of fish-tissue decomposition such as trimethylamine *(13, 14, 26, 28)*, ammonia *(21, 28, 31)*, volatile acids *(14, 17, 26)*, indole and skatole *(12, 29)*, and others *(1, 3, 4, 8, 14, 15, 24, 26, 30)*. In general, the content of these substances has been found to increase in fishery products undergoing spoilage. Whether these increases are measurable and indicate the degree of spoilage during an early stage or indicate the onset of spoilage before it can be detected organoleptically, however, is questionable.

It would seem that a test to measure the disappearance of constituent substances of fish tissues during spoilage perhaps would be a more logical basis for such an index. These substances might disappear in a more gradient manner than the intermediate or end products might form, at least during initial stages of decomposition, since numerous and complex pathways may be involved in the decomposition of the constituent substances and in the formation of the ultimate products.

It was considered of value in the present study, therefore, to find some substance in fish tissue that could be utilized in a critical experiment to evaluate the approach to spoilage indices proposed here. Cystine was chosen for study since the S-S bonds joining peptide chains are quite labile and since results from chemical studies have indicated that hydrogen sulfide *(1, 26, 27)* and sulfhydryl-containing compounds *(3, 18, 25)* increase in fishery products undergoing spoilage.

The reported increases in the content of sulfhydryl compounds during spoilage indicated the desirability also of determining a change in the total sulfhydryl content of unspoiled and of spoiled fish tissue. Any decrease in the content of cystine then might be correlated with an increase in the content of total sulfhydryl groups during spoilage. It also would be desirable to determine the total nitrogen, free ammoniacal nitrogen, and total aerobic bacterial plate count in the unspoiled and spoiled fish tissue to provide further indications of spoilage and clearer insight into the spoilage processes.

Shrimp were chosen for study since staff at this laboratory are engaged in research pertaining to the development of standards of grades for shrimp and shrimp products. A satisfactory quality index of shrimp would aid in the objective division of distinct grades of quality of this natural resource.

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* Deceased.

The objective of the present study, therefore, was to determine changes in total nitrogen, free ammoniacal nitrogen, total aerobic bacterial plate count, total sulfhydryl groups, and cystine in unspoiled and in spoiled shrimp in order to evaluate the hypothesis that a test to measure the disappearance of a constituent unit of fresh fish tissue would be a more sensitive chemical index of quality than a test to measure an increase of intermediate and end products during spoilage. A possible decrease in the content of cystine might be such an index of shrimp quality.

PROCEDURES AND RESULTS

Shrimp samples. The shrimp used in this study were the commercial pink variety *(Penaeus duorarum)*, caught near the Tortugas Islands in the Gulf of Mexico. They were of medium size, ranging from 28–32 shrimp per lb. The shrimp were frozen immediately upon landing and sent by air express to this laboratory, where they were stored at −15° C in moisture-proof containers. Testing was begun immediately.

Initially, cystine and sulfhydryl analyses were conducted on samples of shrimp selected at random to determine the variability of this lot of shrimp. Differences obtained were not statistically significant ($p = >0.01$) for either cystine or sulfhydryl content. Therefore, the shrimp of this lot were considered to be of a single population.

Samples consisting of 6 shrimp each were selected at random from frozen storage as needed. One sample was stored at 2° C for 18 days. A second sample was removed from frozen storage 18 days later and then thawed at 2° C for 6 hr. Thus, the various chemical analyses could be conducted simultaneously on one sample of the freshly thawed unspoiled shrimp and on one sample of the 18-day spoiled shrimp. The samples of freshly thawed, unspoiled shrimp will be termed "control" shrimp throughout the remainder of the paper. The shrimp stored at 2° C for 18 days were definitely spoiled, since they had off-odor, off-flavor, dehydration of tissues, and a sticky feel.

All analyses were conducted during a 6-month period, so the samples of shrimp analyzed at the end of the study were in frozen storage 6 months longer than were those analyzed at the beginning of the study. The removal of samples from frozen storage at 1½ month intervals was necessary to supply the proper samples of unspoiled shrimp for control. Data obtained from storage studies conducted at this laboratory in connection with development of standards indicate that shrimp are of good quality during frozen storage for 8–9 months if held in moisture-proof containers. Therefore, all of the frozen shrimp used as controls in the present study were considered to be of similar quality.

Total nitrogen and ammoniacal nitrogen. Samples of control and of spoiled shrimp, after being thawed, were peeled, deveined, and macerated in a Waring blender. The total nitrogen content of aliquots of the macerated shrimp was determined in triplicate by the Kjeldahl method; the ammoniacal nitrogen content, in triplicate by the official analytical method of the Norwegian Agricultural Chemical Control Centers as described by Moen *(21)*; and the moisture content, in quadruplicate.

The mean total nitrogen content on a dry weight basis was 14.1% for the control and was 14.5% for the spoiled samples of shrimp (Table 1). This difference was not significant ($p = >0.05$). The mean free ammoniacal nitrogen content increased from 1.9 mg% for the control to 10.6 mg% for the spoiled samples (Table 1). This increase was highly significant ($p = <0.01$), indicating some degree of decomposition of nitrogen-containing compounds.

Bacterial plate counts. During the sixth month total aerobic bacterial plate counts were conducted to obtain quantitative data on the relative numbers of bacteria in the control and the spoiled shrimp. Two 20-g samples each of the control and spoiled shrimp were blended, under aseptic conditions, with 180 ml of buffered water used for dilution according to the method described by the American Public Health Association *(2)*. Tenfold serial dilutions were prepared from this 1-to-10 dilution. One ml of each of the serial dilutions was plated in triplicate on Nutrient Agar—1.5% NaCl.[b] Each of the plates was incubated at 30° C, and those plates containing

[b] Baltimore Biological Laboratories.

TABLE 1

Data on the moisture, total nitrogen and free ammoniacal nitrogen content of freshly thawed unspoiled and spoiled[1] shrimp

Sample pairs[2]	Dry matter		Total nitrogen[3]		Free ammoniacal nitrogen[3]	
	Unspoiled	Spoiled	Unspoiled	Spoiled	Unspoiled	Spoiled
	%	%	%	%	mg %	mg %
A,A'[4]	19.7	20.6	14.4	15.3	2.5	10.4
B,B'	20.3	20.7	14.2	14.9	0.9	11.0
C,C'	20.8	23.6	13.7	14.3	0.8	10.9
D,D'	19.9	21.3	14.4	14.0	2.4	10.6
E,E'	20.7	22.5	14.0	14.3	3.0	10.2
Mean	20.3 ± 0.2[5]	21.7 ± 0.6	14.1 ± 0.1	14.5 ± 0.2	1.9 ± 0.5	10.6 ± 0.1

[1] Samples allowed to spoil for 18 days at 2° C.
[2] The sample pair A,A' (one sample each of the control and spoiled shrimp) was removed from frozen storage and analyzed upon receipt of the lot of shrimp. The remaining sample pairs were analyzed at 1½-month intervals. Thus, sample pair E,E' was analyzed after 6 months of frozen storage.
[3] Calculated as a percent of dry matter.
[4] Data reported as means of quadruplicate analyses of each sample for determinations of dry matter and triplicate analyses of each sample for determinations of total nitrogen and free ammoniacal nitrogen.
[5] Average standard error of the mean.

between 30 and 300 colonies were counted after 48 hr with the aid of a Quebec Colony Counter.

The bacterial population increased from a mean of 0.913 million per g in the samples of control shrimp to 443 million per g in the samples of spoiled shrimp, an increase of almost 500 times.

Sulfhydryl and cystine. Aliquots of approximately 5 g each of both the control and the spoiled samples were hydrolyzed by refluxing with 20% hydrochloric acid for 16 hr at 120° to 125° C. After hydrolysis, the aliquots were diluted in half with resin-distilled water and decolorized with activated charcoal. The hydrolysates were filtered and washed with warm 0.1 *N* hydrochloric acid. The pH of the filtrates and washings was adjusted to 1.0 with sodium hydroxide. These filtrates and washings then were diluted to 50 ml with 0.1 *N* hydrochloric acid.

Sulfhydryl. Two 10 ml aliquots of each of the hydrolysates were analyzed each in quadruplicate, for total sulfhydryl content according to the iodometric titration procedure of Okuda *(22, 23)*. In this procedure, cystine is reduced and is measured as cysteine along with other compounds containing a free sulfhydryl group such as mercaptans and glutathione. The method is not highly specific for sulfhydryls in that other compounds of strong reducing capacity, such as reductone and ascorbic acid, also are measured. The results are reported as grams of total sulfhydryl per hundred grams of total solids (percent) and per hundred grams of nitrogen content of total solids (percent). A known cystine solution was used as a reference standard.

The mean total sulfhydryl contents of samples of control and of spoiled shrimp were 0.70% and 0.90% calculated on a dry-weight basis and 4.96% and 6.22% calculated on a total nitrogen basis, respectively (Table 2). This increase in total sulfhydryl content of the spoiled shrimp is statistically significant ($p = <0.01$).

Cystine. Four 1-ml aliquots of hydrolysates of the control shrimp and four of the spoiled shrimp were used to determine the cystine content according to the method of Sullivan as described by Hess *(16)*, with one modification. This modification consisted of adding each reagent required in the procedure to the individual aliquots at accurately controlled intervals of time. In this method 1,2-naphthoquinone 4-sodium sulfonate is employed to develop color in an alkaline reducing medium. The test is highly specific for the determination of cysteine as reduced cystine. A known solution of cystine was utilized as a reference standard. Results are reported as grams of cystine per hundred grams of total solids (percent) and per hundred grams of the nitrogen content of total solids (percent). Any possible free cysteine that might exist in the shrimp tissue necessarily would be oxidized to cystine or reported as cystine by using these experimental procedures of hydrolyses and testing as described.

TABLE 2

Data on total sulfhydryl and cystine content of freshly thawed unspoiled and spoiled[1] shrimp

Sample pairs[2]	Total sulfhydryl based on dry matter[3]		Cystine based on dry matter		Total sulfhydryl based on nitrogen content of total solids		Cystine based on nitrogen content of total solids	
	Unspoiled	Spoiled	Unspoiled	Spoiled	Unspoiled	Spoiled	Unspoiled	Spoiled
	%	%	%	%	%	%	%	%
A,A'[4]	0.70	0.81	0.64	0.78	4.95	5.31	4.45	5.10
B,B'	0.70	0.83	0.66	0.73	4.94	5.65	4.65	5.03
C,C'	0.75	0.88	0.67	0.88	5.50	6.13	4.94	6.13
D,D'	0.71	0.97	0.64	0.94	4.93	6.95	4.46	6.70
E,E'	0.63	1.00	0.56	1.02	4.48	7.05	3.85	7.16
Mean	0.70 ± 0.02[5]	0.90 ± 0.04	0.63 ± 0.02	0.87 ± 0.05	4.96 ± 0.16	6.22 ± 0.35	4.47 ± 0.18	6.02 ± 0.42

[1] Samples allowed to spoil for 18 days at 2° C.

[2] The sample pair A,A' (one sample each of the control and spoiled shrimp) was removed from frozen storage and analyzed upon receipt of the lot of shrimp. The remaining sample pairs were analyzed at 1½-month intervals. Thus, sample pair E,E' was analyzed after 6 months of frozen storage.

[3] Percent total sulfhydryl is calculated from a cystine standard.

[4] Data reported as means of quadruplicate analysis of two aliquots of each sample for determinations of total sulfhydryl and as means of duplicate analysis of two aliquots of each sample for determinations of cystine.

[5] Average standard error of the mean.

The mean cystine content of samples of the control and of the spoiled shrimp was 0.63% and 0.87% calculated on dry-weight basis and was 4.47% and 6.02% calculated on a total nitrogen basis, respectively (Table 2). This increase in total cystine content of the spoiled shrimp is statistically significant ($p = <0.01$).

DISCUSSION

The increased amount of sulfhydryl compounds occurring during spoilage has been interpreted *(3, 18, 25)* to indicate protein denaturation and decomposition. Many compounds of low molecular weight are formed from the breakdown of proteins containing sulfur *(10)*, and often have been suggested as the source of off-odors occurring during spoilage. In no study, however, has the cystine or other sulfur-bearing constituents of the tissues been determined along with the content of sulfhydryl compounds of tissues undergoing spoilage. The results presented here are interpreted to indicate that under the conditions of this experiment, the increase in total sulfhydryl content of spoiled shrimp can be entirely accounted for by a concomitant increase in total cystine content, which increased, incidentally, oppositely to what was expected.

Off-odors of the shrimp at this stage of spoilage probably are not due in any part to an increase of mercaptans, since there was no increase of sulfhydryl compounds that could not be accounted for by the increase of cystine. Also, there was no apparent decomposition of the protein as a whole, at least at this stage of spoilage. The latter concept also has been arrived at through a study of the bacterial populations of fishery products undergoing spoilage *(11)*.

The fact that the free ammoniacal nitrogen content of the spoiled shrimp was greatly increased as compared to that of the control shrimp indicates a breakdown of some nitrogenous compounds. The increased bacterial population may have been responsible for both of these increases. It has been reported that the contents of other amino acids, particularly histidine *(15, 30)* and tyrosine *(8, 24)*, are increased during spoilage and that the increase of histidine is due to bacterial action *(19, 20)*. It also has been reported that radioactive sulfur when fed to cows and goats in the form of inorganic sulfate is incorporated into the cystine and methionine of the protein of the milk and into the cystine and methionine of the protein of the bacteria of the rumen *(6, 7)*. These results would indicate that bacteria can form cystine, and accordingly, may be responsible for the observed increase noted in this study. The increase in content of free ammoniacal nitrogen, in turn, might be explained by assuming an increase in total metabolic activity, owing to an increasing population of these bacteria. A variation in species of bacteria may have an effect on this increase if other lots of shrimp were tested, however.

Initial results from chromatographic studies conducted at this laboratory indicate that many of the free amino acids found in the control shrimp are greatly reduced in amount or not found at all in spoiled shrimp.

In addition, it has been reported that the α-amino nitrogen content of shrimp *(14)* and of press juice from cod tissue *(5)* decreases during spoilage, although other workers report that the α-amino nitrogen content of shrimp *(9)* and of herring *(26)* increases during spoilage. A measure of α-amino nitrogen is directly correlated with, if not an actual measure of,

free α-amino acids. Assuming that the α-amino nitrogen does decrease, then, the free amino acids could be a source of nitrogenous nutrients for the bacteria and, thus, protein decomposition, *per se*, would not be required to furnish the source of these nutrients. It might also follow that at a stage when all sources of available free nutrient compounds containing nitrogen needed by the bacteria are utilized, decomposition of protein might start, owing to other growth requirements of the bacteria. It was found that the decrease in α-amino nitrogen of press juice from cod tissue approximately equaled an increase in ammoniacal nitrogen, and that this might be due to bacterial deamination of amino acids resulting from protein degradation *(5)*. However, it seems quite possible that the source of amino nitrogen utilized by the bacteria during the early stages of changes accompanying storage of fish very well might be the free amino acids, and only later from the amino acids resulting from protein degradation.

This concept does not rule out the possibility that protein denaturation occurs or that other decomposition products, such as fats and sugars, give the subjective sensations obtained from spoilage products. Conversely, it might suggest that these changes may be the cause of the whole sum of the subjective sensations that are characteristic of this stage of spoilage. Further spoilage, however, would result in decomposition of protein.

These concepts are speculative, of course, for it is entirely possible that the quantity of cystine produced by the bacteria might be so great as to mask the release of cystine from protein in the tissues of the shrimp. Also, the amount of particular mercaptans that are needed to be detected organoleptically may be so small that the increases are not measurable by the method employed and therefore could not be detected chemically in the present work.

Conclusions. The results presented in this report indicate that a measure of decrease in content of cystine of shrimp during spoilage is not a suitable chemical quality index. The work, however, now raises the reverse possibility of using the increase in cystine content as a chemical index of spoilage. The value of the approach to indices proposed in the hypothesis remains unclarified. A test to measure the disappearance of a constituent unit of fresh fish tissue may be a more sensitive chemical index of quality than a test to measure an increase of intermediate or end product during spoilage.

SUMMARY

Results obtained from this study verify those obtained by others, that there is an increase in the total sulfhydryl content of spoiled shrimp. The results further indicate, however, that this increase can be entirely accounted for by the concomitant increase obtained in the cystine content of the spoiled shrimp.

Measurements of free ammoniacal nitrogen content indicate that some degree of decomposition of protein or of other nitrogen-containing compounds has occurred during spoilage.

Determinations of total plate counts indicate that the bacterial population was nearly 500-fold greater in the spoiled than in the unspoiled shrimp.

These results suggest the possibility that the tissue protein of the shrimp may not be decomposing to produce the well-known indications of spoilage,

at least up to the stage that was studied here. The results suggest the possibility that the increased bacterial population may be responsible for the increase in cystine and ammoniacal nitrogen of the spoiled shrimp. Further, the results suggest that protein denaturation; decomposition products, perhaps of fats and sugars; and free nitrogen-containing compounds, such as amino acids; may give the subjective sensations that are attributed to the herein-described stage of spoilage of shrimp.

Although the cystine content during spoilage was shown to increase rather than decrease, as originally supposed, the findings now indicate the possibility of utilizing this increase in cystine content as a chemical index to determine the extent of spoilage of fishery products. However, the failure here to measure a substance that decreases with spoilage does not necessarily invalidate the original concept and purpose of this study, that measuring the disappearance of constituent substances of tissues during spoilage may be a more sensitive chemical index of spoilage than measuring resultant intermediate and end products of decomposition of fish tissue. A suitable substance or substances still may be found.

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TASTE INTERRELATIONSHIPS

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Flavors in food consist of a mixture of tastes and odors accompanied by a variety of oral sensations. Because a flavor formula usually includes two or more of the primary tastes (sour, sweet, salty, bitter), much interest has been shown in the specific action these tastes have on each other. Interactions among taste qualities in foods have been a subject of opinion and speculation with relatively little experimental support. The early literature on the subject of taste interrelationships in aqueous solutions of pure compounds is confusing since conflicting conclusions were obtained from similar experiments as seen in the summary below:

Year	Investigator	Conclusion
1892	Zuntz *(25)*	NaCl increased sweetness of sucrose.
1894	Kiesow *(10)*	NaCl decreased sweetness of sucrose.
1937	Cragg *(6)*	Sucrose decreased sourness of HCl. NaCl had no effect on sourness of HCl.
1943	Fabian *(8)*	NaCl decreased sourness of acids. NaCl increased sweetness of sugars. HCl & acetic acids decreased sweetness of dextose. Acids (except HCl) increased saltiness of NaCl. HCl & acetic acids had no effect on sweetness of sucrose. Lactic, malic, citric, tartaric acids increased sweetness of sucrose. Lactic, malic, acetic, tartaric acids decreased sweetness of fructose. HCl & citric acids had no effect on sweetness of fructose. All sugars decreased saltiness of NaCl. All sugars decreased sourness of acids.
1947	Cameron *(3)*	Urea decreased sweetness of sucrose.
1953	Sjöström *(22)*	1% NaCl decreased sweetness of 3–10% sucrose. 0.5% NaCl increased sweetness of 5–7% sucrose. 1–10% sucrose decreased sourness of 0.04–0.06% acetic acid. 0.04–0.06% acetic acid had no effect on sweetness of 1–5% sucrose. 0.04–0.06% acetic acid decreased sweetness of 6% sucrose and above.
1958	Kamenetzky *(9)*	Sucrose had no effect on the saltiness of sodium chloride. Sucrose significantly decreased the bitterness of caffeine.

Several unrelated investigations have been made on more complex aqueous solutions. There is disagreement as to the influence of ethyl alcohol on apparent sweetness. In one experiment, ethyl alcohol was found to simultaneously decrease acuity of the sense of taste for sucrose and to

convert the sensation of appetite into one of satiety *(13)*. In an investigation of water solutions containing various constituents of wines, ethyl alcohol enhanced the sweetness of sucrose, with no interrelationship between sucrose and acid thresholds *(1)*. Chappell *(5)* reported that sodium chloride not only increased the sweetness of sucrose, but also enhanced the intensity of lemon oil flavor. Also, increasing the lemon oil content caused masking of the taste of salt. In solutions containing organic acids, sucrose, and synthetic raspberry flavoring, judges ascribed greater flavor to sweeter samples as well as greater sweetness to solutions with more flavoring *(23)*.

In processed foods, it is more difficult to ascertain the mechanism by which the primary tastes operate. In an experiment conducted by Mackey and Valassi *(12)*, sucrose, tartaric acid, sodium chloride, and caffeine were easier to detect in liquid than in a foam or gel. Studies on apricot, peach, and pear nectars showed that sucrose enhanced fruit flavor up to an optimum sweetness level beyond which it masked flavor *(24)*. This relationship between sweetness and fruit flavor has been found to be greatly influenced by the acidity of the product *(7, 17, 18, 20, 21, 24)*.

Studies on sugar-flavor relationships have not been limited to sweet foods. The effects of adding sucrose to canned tomato juice were to lessen saltiness, seed flavor, and metallic notes *(4)*. When canned, whole tomatoes were acidified in order to decrease pH to a safer level, it was found necessary to add sucrose to compensate for the sourness and to blend the tomato flavor *(11)*. The effect of adding sucrose during the cooking of cream style corn was a desirable augmentation of sweetness and a reduction of sourness and bitterness. Conversely, when salt was increased in cream style corn, sweetness was decreased *(4)*. In processed *(16)* and genuine *(19)* dill pickles, sub-threshold levels of sucrose improved acceptability by counteracting over-acidity and by enhancing the spicing flavors.

In the present investigation, the taste interactions of sucrose, citric acid, sodium chloride, and caffeine at sub-threshold, threshold, and supra-threshold levels, are described. In addition to studying these interrelationships in aqueous solutions, similar experimentation was carried out with more complex mixtures of fruit nectars and tomato juice.

EXPERIMENTAL METHODS

Five male and 5 female college students between 19 and 25 years of age were employed on a laboratory taste panel which met from 4:30 to 5:30 p.m. Monday through Thursday. Good health, age, interest, and dependability were the only criterion used in selection of judges.

All tests were designed so that 5 judges could serve the other 5 tasters who were seated in individual, partitioned booths equipped with constant lighting, temperature, and humidity. Upon completion of a taste set (6–8 min), judges would alternate sides, thereby reducing fatigue and monotony. Tasters and servers were under constant supervision to assure uniformity of conditions.

Aqueous solutions

To determine the effect of training, the judges evaluated the test sets on their first meeting date. The subsequent 7 meetings were utilized to acquaint the students with the methodology of tasting and to train them to recognize small concentrations of sucrose, caffeine, citric acid, and sodium chloride (reagent grades) in solutions of distilled water.

After the training period, 6 weeks were spent determining individual taste thresholds for the 4 compounds mentioned above *(15)*. The taste identification thresholds (minimum

concentration at which taste could be correctly identified for 2 successive presentations) were then used as a guide for preparation of the solutions for the final taste interrelationship study.

The experiment was designed to determine the influence of:

1. Citric acid, sodium chloride, and caffeine on the sweetness of sucrose.
2. Sucrose, sodium chloride, and caffeine on the sourness of citric acid.
3. Sucrose, citric acid, and caffeine on the saltiness of sodium chloride.
4. Sucrose, citric acid, and sodium chloride on the bitterness of caffeine.

The methodology employed was a modification of the procedure described by Fabian and Blum *(8)*. A test series consisted of 6 samples containing supra-threshold levels of one compound, designated as the "Primary Series." To each of these 6 solutions, a sub-threshold amount of another compound, designated as the "Secondary Compound," was added. The "Standard" consisted of an intermediate level of the primary compound, only. The concentrations within series always varied above and below that of the solution to be matched. There was a sufficient difference in concentration between adjacent solutions within series so that the judges could easily detect the increase in taste. Judges received 100 ml of the marked standard solution and 50 ml of each of the 6 samples in the series. Each series was presented in random order and the judges had no information as to the identity of the secondary compound.

A sample score card follows:

SET_____

NAME_____ DATE_____

The beaker marked "S" contains sucrose. Taste each of the 6 coded solutions, then circle the number of the sample which has the same sweetness as "S".

CODE	1	2	3	4	5	6
BEAKER						

If the match were made at a lower concentration than the standard, the secondary taste was considered to enhance the effect of the primary taste. If the match were made at a higher concentration than the standard, the secondary taste was considered to reduce the intensity of the primary taste. This latter phenomenon is referred to by Fabian and Blum *(8)* as "compensation."

Freshly distilled water was used in the preparation of the test solutions. Fountains in the tasting booths provided for expectoration and only freshly distilled water was allowed for oral rinsing at the taster's discretion. All fluids were freshly prepared and maintained at room temperature in coded, glass-stoppered bottles.

Taste interrelationships of all compounds except caffeine (eliminated when judges complained of the fatigue and discomfort of tasting this bitter compound) were determined by 4 groups of panels:

1. Untrained laboratory panel (24 volunteers with little or no panel experience).
2. Untrained students (10 paid tasters; first session).
3. Trained students (same as No. 2, after 8 weeks' experience).
4. Trained students (same as No. 3, after 1 month).

Category No. 3 above was included to test the effect of general taste experience, whereas category No. 4 was included to test the effect of specific experience with the matching-type methodology. Degree of enhancement or depression was determined by calculation of the standard deviation and the least significant difference between average values.

Fruit nectars

The same student panel was utilized to determine the effect of sucrose and acid variations in canned apricot and pear nectars on accuracy of matching a standard within a randomized series. The nectars used consisted of 55% fruit and 45% water by weight with no added syrup. Pear nectars of 2 natural acidity levels were used (0.130% and 0.158% acid calculated as citric acid anhydride). The apricot nectar contained a natural total acidity of 0.590%.

Sucrose at levels of 4, 6, 8 and 10% by weight was added to the apricot nectar. Six percent sucrose was added to the pear nectar to make it more palatable. These sweetened mixtures were then used as bases to which were added varying levels of citric acid, sodium chloride and additional sucrose. The same methodology of tasting as described for the aqueous solutions was used, wherein the judges were required to match the sample within a randomly presented series with a designated standard.

Tomato juice

The effect of the addition of different combinations of sucrose, sodium chloride, citric acid and caffeine on the acceptability of canned tomato juice with a natural total acidity of 0.370% was determined also. Five coded samples, including a hidden control, were evaluated per session using the following score card:

SET_____

NAME_____ DATE_____

BEAKER		MORE ACCEPTABLE				LESS ACCEPTABLE			
NUMBER	SAME	SLIGHT	MODERATE	LARGE	EXTREME	SLIGHT	MODERATE	LARGE	EXTREME

Judges were required to indicate whether the treated samples were the same, more, or less acceptable than the marked untreated control. Also, degree of deviation in acceptability from the control was recorded.

RESULTS AND DISCUSSION

Aqueous solutions

Tables 1 through 5 summarize the results obtained from panel evaluation of combinations of primary tastes in distilled water solutions.

Table 1. In general, the effect of adding sub-threshold levels of the secondary compound was depression of the intensity of the standard solutions, i.e., sucrose decreased the sourness of acid; acid decreased the sweetness of sucrose; sucrose decreased the saltiness of sodium chloride, etc. Responses of the untrained laboratory panel differed from those of the untrained student panel only for Set IV. The untrained students indicated that sodium chloride had an enhancing effect on the sweetness of sucrose. After training, the student panel indicated that acid depressed the sweetness of sucrose to an even greater degree. Further training (final column) showed no changes of opinion, despite the fact that the judges increased in taste sensitivity and thereby were measuring the effect of threshold levels

TABLE 1

Interrelationship of citric acid, sucrose and sodium chloride in water solutions

Set	Secondary compound (Sub-threshold)	Standard solution	Primary series[1]						Average values[2, 3]			
			1	2	3	4	5	6	Untrained lab. panel	Untrained students	Trained students	Trained students (1 mo. later)
			(molarity x 10^{-2})						*(24)*	*(16)*	*(16)*	*(20)*
I	1.0 Sucrose	0.3 Acid	0.1	0.2	0.3	0.4	0.5	0.6	3.2	3.5	3.2	3.2
II	0.1 Acid	5.0 Sucrose	1.0	3.0	5.0	7.0	9.0	11.0	3.1	3.6	4.3	3.8
III	1.0 Sucrose	9.0 NaCl	1.0	5.0	9.0	13.0	17.0	21.0	3.2	3.1	3.3	3.4
IV	2.0 NaCl	5.0 Sucrose	1.0	3.0	5.0	7.0	9.0	11.0	3.4	2.5	3.4	3.0
V	0.1 Acid	9.0 NaCl	1.0	5.0	9.0	13.0	17.0	21.0	3.4	3.3	3.8	3.4
VI	2.0 NaCl	0.3 Acid	0.1	0.2	0.3	0.4	0.5	0.6	3.2	3.0	3.3	3.4
L.S.D.[4]									N.S.	0.7	0.8	0.6

[1] Primary series consists of molar amounts of the standard compound. Correct value underlined.
[2] Average value of the primary series: values greater than 3 indicate the secondary compound has a depressing effect on the intensity of the standard; values less than 3 indicate the secondary compound has an enhancing effect on the intensity of the standard.
[3] Values in parentheses indicate number of replications per set.
[4] Least significant difference between average values at the 5% level of probability.

of the secondary compound on the intensity of the standard. For individual thresholds for the 4 primary tastes, the reader is referred to a previous publictaion *(15)*.

The effect of varying: (a) the concentration of the secondary compound, (b) the concentration of the standard, and (c) the increments in the matching series are summarized in Tables 2 through 5.

TABLE 2

Effect of citric acid, sodium chloride and caffeine on sweetness of sucrose

Set	Secondary compound	Sucrose standard	Sucrose series						No. eval.	Average score[1, 2]	L.S.D.[3]
			1	2	3	4	5	6			
			(molarity x 10^{-2})								
I	Acid										
	0.10	5.0	1.0	3.0	5.0	7.0	9.0	11.0	20	3.8	
	0.02	2.4	1.2	1.8	2.4	3.0	3.6	4.2	14	3.0	0.8
	0.01	1.6	0.4	1.0	1.6	2.2	2.8	3.4	12	3.3	
	0.01	2.2	0.4	1.0	1.6	2.2	2.8	3.4	15	4.1[4]	
II	NaCl										
	2.0	5.0	1.0	3.0	5.0	7.0	9.0	11.0	20	3.0	
	0.8	2.4	1.2	1.8	2.4	3.0	3.6	4.2	15	3.1	0.9
	0.5	1.6	0.4	1.0	1.6	2.2	2.8	3.4	13	2.8	
	0.5	2.2	0.4	1.0	1.6	2.2	2.8	3.4	13	3.1[4]	
III	Caffeine										
	0.10	2.4	1.2	1.8	2.4	3.0	3.6	4.2	12	4.2	
	0.08	2.0	0.4	1.2	2.0	2.8	3.6	4.4	12	3.7	N.S.
	0.08	2.5	0.5	1.5	2.5	3.5	4.5	5.5	12	3.5	

[1] Average of the numerical values assigned to the sucrose series: Underlined values indicate the solution in each respective set that matches the sucrose standard.

[2] Values greater than the correct sucrose series number indicate the secondary compound has a depressing effect on the intensity of the standard; values less than the correct series number indicate the secondary compound has an enhancing effect on the intensity of the standard.

[3] Least significant difference between average scores within each set at the 5% level of probability.

[4] Subtract 1.0 from this figure when applying the L.S.D. value.

Table 2. Citric acid at threshold level ($0.001M$), had a definite depressing effect on the sweetness of sucrose whereas sub-threshold levels (0.0002 and $0.0001M$) had only a slight depressing influence. Sodium chloride did not seem to interfere with the sweetness of sucrose except for a depressing effect in 2 cases. Caffeine, at both threshold ($0.001M$) and sub-threshold levels ($0.0008M$) had a great depressing effect on the sweetness of sucrose. It should be pointed out that the panel showed great dislike for threshold and supra-threshold quantities of caffeine. It was not determined, however, to what extent this dislike may have affected performance.

Table 3. All levels of citric acid were depressed in sourness upon the addition of threshold or sub-threshold levels of sucrose, sodium chloride, and caffeine. In general, sucrose had the greatest reducing effect and sodium chloride had the least. The L.S.D. values for Set I suggest that concentration and placement of the correct match within a series influenced the response.

TABLE 3

Effect of sucrose, sodium chloride, and caffeine on sourness of citric acid

Set	Secondary compound	Citric acid standard	Citric acid series						No. eval.	Average score[1, 2]	L.S.D.[3]
			1	2	3	4	5	6			
			(molarity x 10^{-2})								
I	Sucrose										
	1.0	0.30	0.10	0.20	0.30	0.40	0.50	0.60	20	3.2	
	1.5	0.10	0.02	0.06	0.10	0.14	0.18	0.22	13	3.4	0.6
	1.0	0.09	0.01	0.05	0.09	0.13	0.17	0.21	15	3.7	
	1.0	0.13	0.01	0.05	0.09	0.13	0.17	0.21	13	3.9[4]	
II	NaCl										
	2.0	0.30	0.10	0.20	0.30	0.40	0.50	0.60	20	3.4	
	0.8	0.10	0.02	0.06	0.10	0.14	0.18	0.22	14	3.4	N.S.
	0.5	0.09	0.01	0.05	0.09	0.13	0.17	0.21	15	3.4	
	0.5	0.13	0.01	0.05	0.09	0.13	0.17	0.21	17	4.4[4]	
III	Caffeine										
	0.08	0.18	0.02	0.10	0.18	0.26	0.34	0.42	12	3.2	
	0.08	0.11	0.01	0.06	0.11	0.16	0.21	0.26	15	4.0	0.7
	0.10	0.10	0.02	0.06	0.10	0.14	0.18	0.22	14	3.4	

[1] Average of the numerical values assigned to the citric acid series: Underlined values indicate the solution in each respective set that matches the acid standard.

[2] Values greater than the correct citric acid series number indicate the secondary compound has a depressing effect on the intensity of the standard; values less than the correct series number indicate the secondary compound has an enhancing effect on the intensity of the standard.

[3] Least significant difference between average scores within each set at the 5% level of probability.

[4] Subtract 1.0 from this figure when applying the L.S.D. value.

TABLE 4

Effect of sucrose, citric acid, and caffeine on saltiness of sodium chloride

Set	Secondary compound	NaCl standard	Sodium chloride series						No. eval.	Average value[1, 2]
			1	2	3	4	5	6		
			(molarity x 10^{-2})							
I	Sucrose									
	1.0	9.0	1.0	5.0	9.0	13.0	17.0	21.0	20	3.4
	1.5	1.4	0.2	0.8	1.4	2.0	2.6	3.2	14	3.6
	1.0	0.9	0.1	0.5	0.9	1.3	1.7	2.1	14	3.7
	1.0	1.3	0.1	0.5	0.9	1.3	1.7	2.1	14	4.1
II	Acid									
	0.1	9.0	1.0	5.0	9.0	13.0	17.0	21.0	20	3.4
	0.02	1.4	0.2	0.8	1.4	2.0	2.6	3.2	14	3.3
	0.1	0.9	0.1	0.5	0.9	1.3	1.7	2.1	14	3.4
	0.1	1.3	0.1	0.5	0.9	1.3	1.7	2.1	14	3.9
III	Caffeine									
	0.08	1.1	0.1	0.6	1.1	1.6	2.1	2.6	13	3.5
	0.08	1.8	0.2	1.0	1.8	2.6	3.4	4.2	14	3.5
	0.10	1.4	0.2	0.8	1.4	2.0	2.6	3.2	13	3.4

[1] Average of the numerical values assigned to the sodium chloride series: Underlined values indicate the solution in each respective set that matches the standard.

[2] Values greater than the correct sodium chloride series number indicate the secondary compound has a depressing effect on the intensity of the standard; values less than the correct series number indicate the secondary compound has an enhancing effect on the intensity of the standard. No significant differences between average values within sets were observed.

TABLE 5

Effect of sucrose, sodium chloride, and citric acid on bitterness of caffeine

Set	Secondary compound	Caffeine standard	Caffeine series						eval. No.	Average value[1, 2]
			1	2	3	4	5	6		
			(molarity x 10^{-2})							
I	Sucrose									
	1.0	0.13	0.01	0.07	0.13	0.19	0.25	0.31	13	3.3
	1.0	0.18	0.02	0.10	0.18	0.26	0.34	0.42	13	3.3
	1.5	0.10	0.02	0.06	0.10	0.14	0.18	0.22	15	3.9
II	NaCl									
	0.7	0.13	0.01	0.07	0.13	0.19	0.25	0.31	12	3.2
	0.7	0.18	0.02	0.10	0.18	0.26	0.34	0.42	14	3.7
	0.8	0.10	0.02	0.06	0.10	0.14	0.18	0.22	15	3.5
III	Acid									
	0.01	0.13	0.01	0.07	0.13	0.19	0.25	0.31	12	3.3
	0.01	0.18	0.02	0.10	0.18	0.26	0.34	0.42	19	3.3
	0.02	0.10	0.02	0.06	0.10	0.14	0.18	0.22	15	3.3

[1] Average of the numerical values assigned to the caffeine series: Underlined values indicate the solution in each respective set that matches the standard.

[2] Values greater than the correct caffeine series number indicate the secondary compound has a depressing effect on the intensity of the standard; values less than the correct series number indicate the secondary compound has an enhancing effect on the intensity of the standard. No significant differences between average values within sets were observed.

Table 4. Sucrose, citric acid, and caffeine reduced the saltiness of sodium chloride to approximately an equal extent. Neither concentration nor placement of the correct match significantly affected the average values obtained.

Table 5. Without exception, the bitterness of caffeine was reduced by sucrose, sodium chloride, and citric acid. Increasing the concentration of the sucrose standard further depressed the bitterness of the caffeine solutions, but not to a significant degree.

These results agree with Cameron's statement, "The stimulus of a sweet solution to 'sweet' taste-buds may be depressed by a concomitant non-sweet stimulus to the same taste-buds, so that a lesser intensity of stimulus is registered" *(3)*. This theory could be extended to include all

TABLE 6

Interrelationship of citric acid, sucrose and sodium chloride in apricot nectar

Set	Standard	Matching series[1]						Average values[2]				L.S.D.[4]
		1	2	3	4	5	6	10	8	6	4	
		(percentage)						% Sucrose[3]				
I	Citric acid 0.090%	0	.030	.060	.090	.120	.150	3.3	4.7	4.0	4.1	0.2
II	Sucrose 3.6%	0	1.2	2.4	3.6	4.8	6.0	3.9	4.0	3.7	3.8	0.1
III	NaCl 0.207%	0	.067	.140	.207	.274	.341	4.1	4.8	4.3	4.0	0.7

[1] Series consist of increasing amounts of standard compound. Correct value underlined.
[2] Average series number (Ten replications).
[3] Amount added to all samples within respective series prior to addition of other compound.
[4] Least significant difference between average scores within each set at the 5% level of probability.

the primary tastes, as the present data indicate. However, more information regardnig the effect of concentration of the compounds on taste response is needed.

Fruit nectar

Table 6 shows the extent to which varying amounts of sucrose (4, 6, 8, 10%) in apricot nectar influenced the judges' ability to match the standard within each respective series. In most cases, the lower the sucrose level, the greater ease the panel had in matching the correct citric acid standard (Set I). This indicates, once again, that sucrose depresses the sourness of acid. Set II shows that varying the sucrose content affected the judges' ability to match the standard to the correct sample within the series. The presence of sucrose in Set III had little effect on matching of the salt standard except for the high value of 4.8 in the case of 6% sucrose.

In Table 7, the influence of citric acid, sucrose, and sodium chloride on accuracy of matching the standard within each series of pear nectar is summarized. When a pear nectar of low natural acidity (PN-1) was used as the base solution, there was a tendency for the panel to match the citric acid, sucrose, and sodium chloride standards slightly below the correct con-

TABLE 7

Interrelationship of citric acid, sucrose and sodium chloride in pear nectar

Set	Standard	Matching series[1]						Average values[2]		
		1	2	3	4	5	6	PN-1[3]	PN-2[4]	L.S.D.[5]
		(percentage)								
I	Citric acid									
	0.090%	0	.045	.090	.135	.180	.225	2.9	3.2	
	0.135	0	.045	.090	.135	.180	.225	3.7	3.8[6]	
	0.075	0	.036	.075	.111	.147	.183	.....	3.1	0.6
	0.111	0	.036	.075	.111	.147	.183	.....	4.4[6]	
	0.061	0	.029	.061	.090	.119	.148	.....	3.1	
	0.090	0	.029	.061	.090	.119	.148	.....	4.3[6]	
II	Sucrose									
	4.0%	0	2.0	4.0	6.0	8.0	10.0	2.7	3.1	
	6.0	0	2.0	4.0	6.0	8.0	10.0	3.8	4.4[6]	
	3.2	0	1.6	3.2	4.8	6.4	8.0	.....	3.5	0.8
	4.8	0	1.6	3.2	4.8	6.4	8.0	.....	4.2[6]	
	2.4	0	1.2	2.4	3.6	4.8	6.0	.....	3.0	
	3.6	0	1.2	2.4	3.6	4.8	6.0	.....	3.7[6]	
III	NaCl									
	0.222%	0	.111	.222	.333	.444	.555	2.9	3.4	
	0.333	0	.111	.222	.333	.444	.555	4.1	4.2[6]	
	0.188	0	.085	.188	.273	.358	.443	.....	2.9	0.6
	0.273	0	.085	.188	.273	.358	.443	.....	4.2[6]	
	0.140	0	.067	.140	.207	.274	.341	.....	3.6	
	0.207	0	.067	.140	.207	.274	.341	.....	4.0[6]	

[1] Series consist of increasing amounts of standard compound. Correct value underlined.
[2] Average series number (Ten replications).
[3] Pear nectar with low natural acidity (0.130%). No significant differences between average values.
[4] Pear nectar with high natural acidity (0.158%).
[5] Least significant difference between average scores within each set at the 5% level of probability.
[6] Subtract 1.0 from this figure when applying the L.S.D. value.

centration within each respective series. However, when pear nectar of higher natural acidity (PN-2) was used as the base solution, the panel tended to match the standard to a concentration slightly above the correct level within the respective series. This suggests that the greater the acidity the greater the depressing effect on the intensity of the added compound.

The effect of position of the correct match within series was tested, i.e., in one group the correct match was series number 3 whereas in the following group, using the same increments, the correct match was series number 4. The results showed that the placement of the matching sample in the series had no significant effect on the judges' selection except for Series 5 and 6 within Set III. In general, judges matched the standard with samples of slightly higher concentration within the various series indicating a slight depressing effect of the secondary compound.

Tomato juice

Additions of threshold and supra-threshold levels of sucrose, citric acid, sodium chloride, and caffeine decreased the acceptability of canned tomato juice. Although 80 various combinations of the 4 compounds were tasted, only the following 6 combinations were considered to improve the acceptability of the juice:

Compound	Combinations					
	1	2	3	4	5	6
	% added (w/v)					
Sucrose	0	0	0.12	0.15	0.10	0.10
Sodium Chloride	0.50	0.25	0	0	0.50	0.50
Citric Acid	0.05	0.03	0.08	0.09	0.03	0.03
Caffeine	0	0	0	0	0	0.01

In combinations 1 and 2 the salt-acid ratio is approximately 10. In combinations 3 and 4 the sucrose-acid ratios are 1.50 and 1.66, respectively. Combination 5 shows the proportions of sucrose, sodium chloride, and citric acid considered to improve tomato juice flavor. Caffeine alone did not improve acceptability, however in the amount shown in combination 6, the juice was considered superior to the untreated control. Apparently, this level of caffeine (0.01%) was sub-threshold or was minimized by the other compounds.

SUMMARY

The taste interrelationships of sub-threshold, threshold, and supra-threshold concentrations of sucrose, citric acid, sodium chloride, and caffeine in aqueous solutions were determined by 10 highly trained tasters. In general, all compounds were found to depress the intensity of each other. The most pronounced effect was the reduction of the sweetness of sucrose by citric acid and the reduction of the sourness of citric acid by sucrose. Increasing the sugar or acid content of apricot and pear nectars decreased the accuracy with which the judges could match the taste of a standard with a randomized series. In tomato juice, any of the compounds alone decreased acceptability; however, the proper proportion of all 4 compounds resulted in a juice superior in acceptability to an untreated control.

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APPLICATION OF THE PAIRED COMPARISON METHOD TO THE STUDY OF FLAVOR DIFFERENCES IN COOKED VEGETABLES[a]

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The choice of procedures for evaluating flavor requires a decision as to the purpose of the experiment: whether (1) consumer preferences or (2) differences in a specific flavor attribute are to be determined. For the first purpose, the opinion of a wide sampling of consumers is ultimately required; for the second, trained laboratory panels can give a measure of differences in a flavor attribute without necessarily stating a preference.

The present study is of the second type; the paired comparison method of Bradley and Terry *(2, 6)* was used to determine which methods of cooking developed strong flavors in vegetables of the cabbage family. In addition, it was used to investigate certain aspects of panel performance: namely, the homogeneity of evaluations of flavor differences, the sensitivity of the panel to the flavor attribute under study, and the trends during replication which might be indicative of changes in performance within a given series.

METHOD

Preparation of vegetables. Cabbage, cauliflower, and broccoli were obtained through regular market channels. Sampling was done in such a way as to insure as uniform portions as possible while maintaining a preparation typical of the particular vegetable. Cabbage was shredded; cauliflower was separated into flowerets; and broccoli was cut into 5 in lengths. Enough vegetable was cooked in each case to yield 4 one-half cup servings as defined by Halliday and Noble *(5)*. If more vegetable was required for judging, duplicate or triplicate cookings were prepared and combined after cooking.

Cooking methods. The cooking methods used were those described by Halliday and Noble *(5)* for cooking in boiling water, in a pressure saucepan, in a steamer, and in a tightly covered saucepan using the minimum amount of water to prevent scorching. Cooking times were such as to yield equal tenderness in samples cooked by different methods.

Training of judges. Cabbage cooked in boiling water until tender, in a "waterless" saucepan in 72 ml of water for 50 min, and 1:1 mixtures of these was used in training sessions. At the first session, samples from both methods of cooking were presented to the judges. The flavors were discussed and described as *mild* in cabbage cooked in boiling water and *strong and hay-like* in overcooked cabbage. At following training sessions, samples cooked by the 2 methods and also 1:1 mixtures of them were presented to the judges in pairs as will be described subsequently. At the conclusion of each judging, the flavors were again discussed and those judges who made errors in ordering samples retasted the samples.

Studies with cabbage mixtures. Mixtures of cabbage cooked in boiling water and in the "waterless" saucepan in proportions up to 16 parts by weight of the former to 1 part of the latter were used in preliminary studies to determine the sensitivity of the panel to the specific flavor, the homogeneity of panel performance, and the homogeneity of replications. Samples were presented to the judges in pairs as described in the following section.

Judging of samples for flavor differences. Six college students served as judges. Judging was done in semi-isolated booths in a room separated from the preparation area. Red lighting was used to eliminate color differences.

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Each judge was given a set of paired samples and asked to indicate on a score card which sample in each pair had the stronger flavor. Each set consisted of a sample from each method of cooking or from each mixture paired with one from every other method of cooking or mixture. Thus, in the studies of 4 methods of cooking, each set consisted of 6 pairs of samples. In studies of cabbage mixtures, in which samples from 2 different mixtures were compared with samples cooked in boiling water, each set consisted of 3 pairs of samples. The order of presentation of the pairs and of samples within pairs was randomized for each judge. The judges were required to taste the samples in the order in which they were presented and, when necessary, to retaste in the original order. In addition, they were required to make a decision about a given pair before tasting the next pair. At the conclusion of each judging, the judges were told whether their choices agreed with those expected on the basis of the specific hypothesis being tested.

At least four separate replicate judgings were made for each vegetable.

Analysis of judging results. The choices of judges were translated into scores of 1 for the sample with the stronger flavor and 2 for the other member of the pair. These scores were analyzed by methods suggested by Terry, Bradley, and Davis *(6)*. In most instances, the number of replications exceeded those tabulated by Bradley and Terry *(2)* so that estimates of p_i and B_i were calculated directly from the appropriate equations.

The test of agreement proposed by Bradley and Terry was used to determine whether the performances of the judges were homogeneous. This test is based on the difference between the pooled likelihood ratio statistic (B_1) and the combined likelihood ratio statistic (B_1^c). In the former statistic, the scores of individual judges are pooled; thus, each judge serves as a replication of the basic design. In the latter statistic, the identity of each judge with his scores is maintained throughout the calculation. The test also served as the basis for the choice of the method to be used in testing the significance of treatment differences; i.e., whether tests of treatment differences should be based on pooled or combined results. The former method is possible if the panel is homogeneous; the latter, if it is non-homogeneous.

The test of agreement was also used to determine whether replications of a given series of cabbage mixtures showed consistent results. For this purpose, the data were grouped by replications instead of judges as was the case in the use of the test in studying the homogeneity of the panel.

The significance of individual comparisons among treatments was tested by the sign test of Dixon and Massey *(3)*, since Bradley *(1)* has suggested that in such comparisons the test procedures reduce to binomial or sign test procedures. One-sided tests of significance were used since it was dseired to test whether a given cooking method resulted in a milder flavor than another method or group of methods.

RESULTS AND DISCUSSION

Studies with cabbage mixtures. Judges' scores and certain statistics calculated from them for mixtures of cabbage cooked in boiling water until tender and overcooked in a "waterless" saucepan are given in Table 1.

None of the values of X^2 calculated for the test of agreement among judges exceeded that for the 5% level of significance. Therefore, it was concluded that this panel was functioning as a homogeneous unit and that tests of treatment differences could be based on the pooled likelihood ratio statistic (B_1).

The advantage of a homogeneous panel is demonstrated particularly well in the fourth series. The value of X_2^2 associated with the test of treatment differences based on the pooled likelihood ratio statistic, B_1, equalled 14.15 (Table 1) and falls between the 0.1% and 0.05% levels of significance. The value of X_{12}^2 associated with the test based on the combined likelihood ratio statistic, B_1^c, equalled 25.97 and falls, therefore, between the 1% and 5% levels of significance.

The values of X_2^2 associated with the test of treatment differences based on pooled data in the other series of cabbage mixtures also exceeded that

TABLE 2

Judges' scores for vegetables cooked by different methods

Vegetable	Method of cooking	Total score[1]	Pooled likelihood ratio statistic (B_1)	X^2 for test of treatment differences[2]
Cabbage	Boiling water	143	23.74	90.31
	Pressure saucepan	99		
	Steamer	97		
	Tightly covered	93		
Cauliflower	Boiling water	122	22.73	70.00
	Pressure saucepan	89		
	Steamer	93		
	Tightly covered	74		
Broccoli	Boiling water	127	24.82	68.73
	Pressure saucepan	98		
	Steamer	93		
	Tightly covered	78		

[1] Perfect scores, assuming the following order of strongness, B.W. <P.S. <St. <T.C.: 144, 96, 72, 48 for cabbage; 126, 84, 63, 42 for cauliflower; 132, 88, 66, 44 for broccoli.
[2] $X^2 > 7.81$ indicates treatment differences significant at 5% level.

for the 5% level of significance. Thus, the panel was able to detect significant differences among flavors of the samples included in each series.

In the first 3 series, the sample containing the smaller amount of overcooked cabbage was selected as having a milder flavor than either of the other two samples a significant number of times. For example, in the first series, samples cooked in boiling water were judged milder than the 2:1 mixture 34 times in 36 replications and the latter was judged milder than the 1:1 mixture 23 times in 36 replications. In the fourth series, the boiling water samples were judged milder than the 8:1 mixture in 19 out of 24 replications, a sufficient number to indicate that the former was significantly milder than the latter. However, both in the comparison of boiling water samples with the 16:1 mixture and the comparison of 16:1 and 8:1 mixtures, the sample containing the smaller amount of overcooked cabbage, that is, boiling water samples in the first instance and 16:1 in the second, was selected as the milder flavored sample in only 16 out of 24 replications, a number insufficient to establish significant differences in the intensity of the flavor. Consequently, it was concluded that, for the judging panel being used, the 16:1 mixture represented the level of intensity of the strong hay-like flavor which could not be distinguished from the mild flavor of cabbage cooked in boiling water.

The values of X^2 associated with the test of agreement calculated for the data grouped by replications in the first two series, 21.23 and 15.69, exceeded the levels of significance for the 5% level, 18.3 and 12.6 for 6 and 4 trials respectively. Those for the last two series, 5.99 and 8.10, did not exceed that for the 5% level of significance for 4 trials. Thus, replicate variability appeared to be present in the first two but not the last two series. However, in no replication in either of the first two series were the values of B_1 large enough to indicate, on the basis of the probabilities associated with them, that the judges were failing to recognize treatment differences. For example, the variability in the second series resulted from 3, 2, 2, 0 incorrect judgments, respectively, in each of the four sets of 18 judgings.

Flavor differences in vegetables cooked by different methods. Judges' scores and certain statistics calculated from them for vegetables cooked in boiling water and by steaming methods are shown in Table 2. The values of X^2 associated with the test of treatment differences show that significant differences were present among the cooking methods. However, as pointed out in studies with cabbage mixtures, this test by itself does not define which of the cooking methods were responsible for these differences.

Individual comparisons of cooking methods followed the pattern of orthogonal comparisons used in the analysis of percentage retentions of ascorbic acid in vegetables cooked by the same methods *(4)*. The flavor of vegetables cooked in boiling water was compared with that of samples cooked by the three steaming methods. Within the steaming methods, the flavor of samples cooked in the pressure saucepan was compared with that of samples cooked in the steamer and tightly covered saucepan to determine whether the high temperature and consequently short cooking times of the pressure saucepan resulted in a milder flavor than did the conditions of the other steaming methods. Finally, the flavor of samples cooked in the steamer was compared with that of samples cooked in the tightly covered saucepan.

Vegetables cooked in boiling water were judged milder in flavor than those cooked by steaming methods in 71 out of 72 judgments for cabbage, 59 out of 63 judgments for cauliflower, and 61 out of 66 judgments for broccoli. It is apparent without statistical treatment of these data that the vegetables were milder in flavor when cooked in boiling water than when cooked by steaming methods.

Vegetables cooked in the pressure saucepan were judged milder than those cooked in the steamer and tightly covered saucepan in 28 out of 48 judgments for cabbage, 24 out of 42 for cauliflower, and 30 out of 44 for broccoli. Application of the sign test to these data showed that only in broccoli was the number of selections sufficiently large to indicate that pressure cooked broccoli was significantly milder in flavor than samples cooked in the steamer and tightly covered saucepan.

Vegetables cooked in the steamer were judged milder than those cooked in the tightly covered saucepan in 12 out of 24 judgments for cabbage, 16 out of 21 judgments for cauliflower, and 15 out of 22 for broccoli. In cauliflower, but not in cabbage and broccoli, the number of selections was sufficient to conclude, on the basis of the sign test, that samples cooked in the steamer were milder in flavor than those cooked in the tightly covered saucepan.

SUMMARY

Flavor differences in vegetables cooked in boiling water and by steaming methods have been studied by the use of a paired comparison method.

Preliminary studies with cabbage established that the members of the panel were homogeneous in their judgments of flavor differences and that mixtures containing 16 parts of cabbage cooked in boiling water and one part of cabbage overcooked in a "waterless" saucepan represented the level of intensity of the strong, hay-like flavor produced by latter method which could not be differentiated by the panel from the mild flavor produced by the former method.

TABLE 1

Judges' scores and certain test statistics for mixtures of cabbage cooked in boiling water and overcooked in a "waterless" saucepan

Series	Proportion by weight of cabbage cooked in:		Score for judge:[1]						Total score	Combined likelihood ratio statistic (B_1')	Pooled likelihood ratio statistic (B_1)	X^2, for test of agreement[2]	X^2, for test of treatment differences[3]
	Boiling water	"Waterless" saucepan	A	B	C	D	E	F					
1	1	0	21	23	24	23	23	24	138	16.738	19.469	12.58	60.07
	2	1	17	18	16	16	14	16	97				
	1	1	16	13	14	15	17	14	89				
2	1	0	16	16	15	15	16	16	94	5.469	8.530	14.09	60.54
	4	1	12	10	13	12	12	10	69				
	2	1	8	10	8	9	8	10	53				
3	1	0	16	16	16	15	15	15	93	7.483	9.573	9.63	55.74
	8	1	12	11	11	13	11	12	70				
	4	1	8	9	9	8	10	9	53				
4	1	0	14	15	15	13	15	11	83	16.036	18.603	11.82	14.15
	16	1	13	12	12	12	11	12	72				
	8	1	9	9	9	11	10	13	61				

[1] Scores for series 1 are sums of 6 replications; all others are sums of 4 replications. Perfect scores for an individual judge are 24, 18, and 12 for series 1; and 16, 12, and 8 for series 2, 3, and 4.

[2] $X_{10}^2 > 18.3$ indicates disagreement among judges, significant at 5% level.

[3] $X_2^2 > 5.99$ indicates treatment differences, significant at 5% level.

Cabbage, cauliflower, and broccoli were found to be milder in flavor when cooked in boiling water than when cooked in steam. They tended to be quite similar in flavor when cooked by the various steaming methods. For example, only broccoli cooked in the pressure saucepan was milder than that cooked in the steamer and tightly covered saucepan.

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CORRELATION OF TASTE PANEL GRADINGS WITH SALT-EXTRACTABLE PROTEIN OF FROZEN FISH FILLETS

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Frozen fish deteriorate in quality after storage for several months. This is partly due to changes in texture which give the cooked flesh what has been described as a "raggy" or "tough" feel to the palate. Notevarp and Heen *(8)*, the first investigators to use taste panel gradings to follow changes in texture of fish flesh during frozen storage, adopted a rating system for comparing frozen material with freshly caught and cooked flesh from the same species. The existence of a relationship between texture of the flesh and the state of the muscle proteins was first suggested by Reay *(9)*, who showed that the proteins became less easily extractable by dilute salt solutions when the fish had been held in the frozen state for some time. Dyer, French and Snow *(4)* showed that this change, which has been considered to be an indication of denaturation, was confined to the structural muscle fiber actomyosin, the protein mainly responsible for the texture of fish muscle.

The relationship between the amount of actomyosin extractable by salt solutions and texture values as measured by taste panels has been studied by several workers, *viz*, Dyer *(2)*; Dyer and Morton *(3)*; Dyer, Morton, Fraser and Bligh *(5)*; and Love *(6)*.

Their variable results show a real need for obtaining and analysing more data before the value of the amount of extractable protein, as an index of texture of the flesh of frozen fish, can be assessed. The experimental work described in this paper was designed with the object of obtaining such data capable of being analysed statistically. The data relate to the ability of a taste panel to detect texture differences in fish held in frozen storage, to the amount of extractable actomyosin, and the correlation between them.

EXPERIMENTAL METHODS

The fish used in this investigation was morwong *(Nemadactylus macropterus)*, a common commercial species. They were from the same catch and of good quality. There were 3 treatments of frozen storage, viz, packed in small cans sealed under a vacuum of 22 in Hg and held at −18° C (vacuum), sealed in M.S.A.T. cellophane bags and held at −18° C and −10° C separately.

To eliminate between-fish variation from experimental error, the treatments were applied to fillets (halves of fish) in a balanced incomplete block design for each of 6 storage times, i.e. $t = 3$, $r = 4$, $b = 6$, $k = 2$, $\lambda = 2$ for each storage time. The 6 storage times were: zero (initial), 1, 2, 3, 4, and 6 months.

The fish were washed and dressed before being halved longitudinally to give fillets. All necessary randomizations (fish to storage times and blocks within storage times, and treatments to fillets) were made. The fillets put into cans were rolled tightly in cylindrical form to minimize the free space. Fillets were treated as individual units in packing. The materials were shelf frozen at −18° C in still air overnight and placed under their respective storage conditions the following morning.

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On removal from storage the fillets were used for the determination of salt-extractable protein and for sensory difference tests for texture. In the case of initial storage time, tests were made within 12 hr of freezing.

At each removal time all material for that period was taken from storage and held at 0° C. The skin was stripped from the frozen fillets; a central longitudinal strip sufficient for nitrogen determinations was removed and the remaining material cut transversely into portions suitable for presentation to tasters. Corresponding transverse portions from the fillets (i.e., treatments) of the same fish were grouped and coded for presentation to individual tasters.

After partial thawing at 0° C for 2 hr, the central strip of each fillet was thawed at room temperature for 30–40 min. A 20–30 g sample of each central strip was extracted with 900 ml of chilled neutral 5% solution of sodium chloride, using a Waring blender and baffle plate as described by Dyer, French and Snow *(4)*. The salt-extracted nitrogen consisted of the freeze-labile actomyosin, the stable albumen and non-protein nitrogen fractions. This nitrogen was determined from an aliquot of the extract by a microkjeldahl method using the modification of McKenzie and Wallace *(7)*. The non-protein nitrogen of the extract was determined on a further aliquot after precipitation of the protein by a 5% trichloroacetic acid solution. Total nitrogen for the central strip of each fillet was measured by the Kjeldahl-Wilfarth-Gunning method of the A.O.A.C. *(1)* on a portion of the mixed sample using at least 2 g for digestion. Salt-extractable protein nitrogen was expressed as a percentage of total protein nitrogen.

Samples cut and coded at 0° C as described, were placed in flowing steam while frozen and cooked for 15 min. They were placed in glass dishes kept warm up to the time of tasting and served to the 12 members of an analytical panel chosen from experienced fish tasters. The tests were paired comparisons where judgment was made on texture, preference going to the less tough. All fillets from one removal time were judged in one session, 3 treatment comparisons being made in succession in random order. Thus the 2 samples in each paired comparison for a given taster were corresponding anatomical portions of fillets of the same fish. Order of presentation of samples within pairs was balanced as far as the number of tasters at a session allowed. Tasters were also asked to indicate their degree of preference as *slight, definite* or *very definite*. A yes-no judgment for texture acceptability was also requested. Taste tests were conducted generally with the usual procedure to eliminate bias and obtain independent judgments.

RESULTS

(a) Determinations of salt-extractable protein nitrogen

Data for salt-extractable protein nitrogen expressed in g per 100 g fish were treated by analysis of variance for storage times separately (Table 1). Variation between fish was quite small but treatment differences occurred as storage proceeded.

To investigate treatment and storage effects further, fillets were taken as experimental units and variation between fish, in view of the results in Table 1, ignored. It was found that, over a period of 1 to 6 months, there was a trend, adequately described as linear, for a decrease in salt-extractable protein nitrogen; this linear rate was not significantly different for the storage treatments (Figure 1), the mean value being a loss of 0.061 g

TABLE 1

Summary of analyses of variance of salt-extractable protein nitrogen (g per 100 g fish)

Source of variation	Degrees of freedom	Mean squares				
		Storage time (months)				
		1	2	3	4	6
Treatments (adjusted for fish)	2	0.0620	0.2054**	0.1850**	0.1890**	0.2356***
Fish (ignoring treatments)	5	0.0733*	0.0390	0.0376	0.0271	0.0114
Intra-fish error	4	0.0188	0.0376	0.0258	0.0211	0.0134
(Pooled error)	20			0.0233		
Total	11					

One, two and three asterisks denote significance at probabilities of 0.05, 0.01, and 0.001 respectively.

per months (∓ 0.012 on 56 degrees of freedom) of salt-extractable protein nitrogen per 100 g fish. The treatments, however, did differ significantly in mean levels; this result is accounted for by assuming that the mean level for the −10° C treatment is lower than those for the other 2 treatments and that the mean level for −18° C treatment is less than that for vacuum ($P = 0.05$ approx). By comparing treatment regressions with the data for initial removal it was found that there was a highly significant decrease ($P < 0.001$) in salt-extractable protein nitrogen during the first month's storage at −10° C; this change was also significant ($P < 0.01$) for the −18° C treatment, but not for vacuum treatment. These results are illustrated in Figure 1. They are also shown in terms of salt-extractable protein nitrogen as percentage of total protein nitrogen, using for the latter the mean of all observations. Total protein nitrogen determinations were constant to a fairly high degree of accuracy for the duration of storage used.

(b) Organoleptic tests

Paired comparison sensory difference tests for texture sometimes resulted in a *no-difference* judgment. For the purpose of significance tests alone these judgments have been ignored and the remaining data examined using a binomial model; the null hypothesis about treatments was tested against the alternate hypothesis that the lower temperature (or the vacuum pack) produced the preferable material.

The data for *initial* removal gave no evidence for rejecting the null hypothesis; this may be regarded as a check on procedure since at this stage the treatments were dummy. The data for subsequent removals were accumulated beginning at one month and are shown in Table 2. Results of the significance tests inserted there show that after 2 months the −10° C treatment was always inferior in texture to both the others which were never significantly different. These results are consistent with those obtained for storage times separately but the accumulated data provide more reliable and sensitive tests.

The no-difference judgments, however, must be regarded as giving some information about the magnitude of the differences and the ability of judges to detect them. Hence, the data, accumulated as before, have been considered from this point of view. The model used supposes that the no-difference judgments constitute a small indefinite proportion of

TABLE 2

Results of paired comparison tests for texture preferences; data accumulated from one month's storage time

Storage time (*months*)	Numbers of judgments					Mean score ± S.D.[1] (approx)	
	Treatment			Total difference	No difference	Total no.	
	Vacuum	−18° C	−10° C				
1	3	4		7	5	12	−0.083
		5	2	7	5	12	0.25 ±0.29
	5		3	8	4	12	0.17
2	7	10		17	7	24	−0.12
		13*	3	16	8	24	0.42* ±0.20
	12*		5	17	7	24	0.29
3	12	15		27	9	36	−0.083
		21**	4	25	11	36	0.47** ±0.17
	20*		7	27	9	36	0.36
4	13	25		38	12	50	−0.24
		28**	8	36	14	50	0.40** ±0.14
	24*		13	37	13	50	0.22
6	23	27		50	12	62	−0.064
		34**	12	46	16	62	0.36** ±0.13
	30*		15	45	17	62	0.24*

[1] Mean score = (number judgments favouring lower temp or 'vacuum' pack − number judgments favouring higher temp or "no vacuum" pack)/total number of judgments ± an approx S.D. of $1/\sqrt{\text{total number judgments}}$.

One and two asterisks denote significance at probabilities of 0.05 and 0.01, respectively.

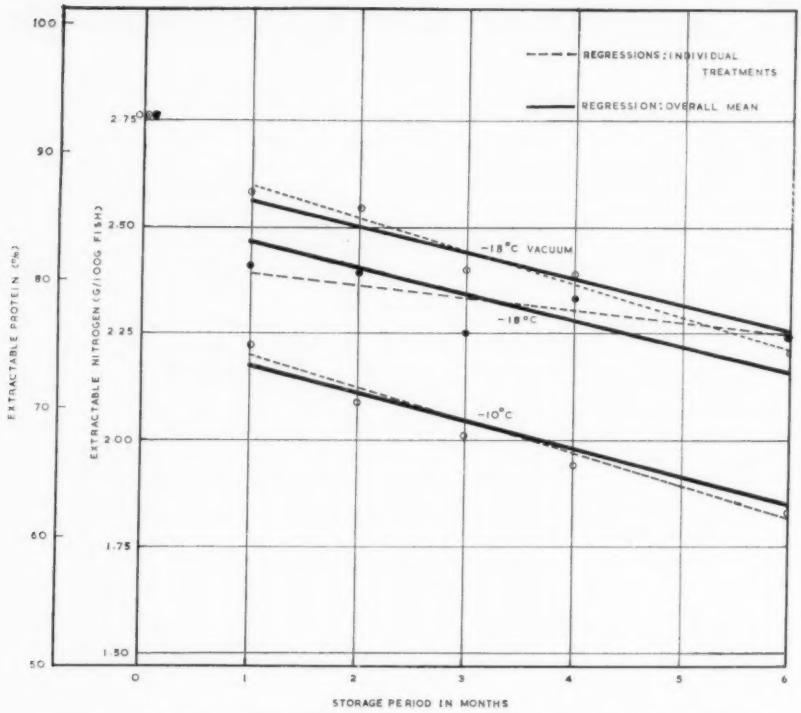

Figure 1. Data for salt-extractable protein nitrogen plotted against storage time. The least squares fits for linear regression on times for period of 1 to 6 months is shown.

the whole and that on the null hypothesis about the treatments the expected proportions of judgments for the treatments are equal; this is tested, as before, against the alternate hypothesis that the lower temperature (or vacuum pack) produced the better material. On this basis the judgments may be regarded as *right, wrong* and *not-different* and arbitrarily scored +1, −1 and 0, respectively; it can be shown that for a large number of judgments and a small proportion of no-difference judgments, the mean score is almost normally distributed about a mean of zero with a standard deviation nearly equal to, but not greater than, $1/\sqrt{N}$ where N is the total number of judgments. Mean scores and their approximate standard deviations so calculated are entered in the last column of Table 2 together with the result of the significance test. These results are identical in nature, except in two instances, with those given previously. Hence, it can be taken generally that at and beyond a storage time of 2 months the −10° C pack is inferior to the other treatments which are not significantly different; the tests ignoring the no-difference judgments appear to be slighly more sensitive, allowance being made for a slight difference in interpretation.

Further to the tests made above, the data for degrees of preference were examined. The degrees of preference slight, definite and very definite were arbitrarily scored as 1, 2, and 3, respectively, (no-difference as 0); the signs of the scores were made positive if they were in accord with the alternate hypothesis made previously. Scores, d, obtained in this way, one for each judgment, were examined by calculating student's t from $t = \overline{d}/\left[S(d-\overline{d})^2/N(N-1)\right]^{1/2}$ with $N-1$ degrees of freedom, N being the total number of judgments. Results obtained by this method on the accumulated data beginning at

one month were consistent with those for storage times separately but gave smaller probabilities; the differences shown to be significant are precisely those shown in Table 2 using the binomial model on the difference judgments only. Hence, analysis of the degrees of preference only served to emphasize the significance obtained otherwise.

Study of the classification of the samples as acceptable or non-acceptable showed that the average proportion rated as unacceptable was nearly one third and these judgments showed the same storage and treatment differences noted above.

DISCUSSION

The results of this experiment suggest that there is a relationship between changes in texture and loss of salt extractability in the proteins of morwong held at −10° C and −18° C for 2 months or longer. Although comparisons were not made between frozen and fresh unfrozen fish, the increase in the proportion of samples with unacceptable texture as storage progressed and the faster rate of change at the higher temperature provided clear evidence of real deterioration in texture.

The tasting panel was unable to detect texture differences between samples after one month's storage when differences between protein extractabilities were significant at a probability of 10% but not at 5%. At all subsequent tastings, when differences in extractability of proteins were significant at the 5% level or better, the panel was clearly able to distinguish texture differences.

Although several workers, Dyer *(2)*, Dyer and Morton *(3)*, Dyer, Morton, Fraser and Bligh *(5)* and Love *(6)* showed that deterioration in texture and loss of salt-extractability of proteins were roughly parallel both for fatty and non-fatty species of fish stored at −10° to −12° C, there is some evidence of departures from this relationship. Dyer *(2)* found a marked fall in the texture of cod held at −12° C for one month and an even more pronounced fall in actomyosin extractability. Dyer and Morton *(3)* working with plaice at −12° C found very little alteration in texture up to 10 weeks' storage although a slight but definite fall in extractability of actomyosin took place during this period. With rosefish at −12° C, Dyer, Morton, Fraser and Bligh *(6)* found a sharp decrease in texture commencing after 15 weeks whereas actomyosin values did not begin to fall sharply until after 21 weeks. Notevarp and Heen *(8)* found that herring and cod held at −9° C showed sharp decreases in texture ratings at the end of one month's storage but these workers did not measure salt extractability of proteins.

At lower temperatures Dyer *(2)* found that Atlantic halibut and cod stored at −23° C showed the same kind of relationship between texture and salt-extractability as at higher temperatures. Dyer, Morton, Fraser and Bligh *(5)* could not, however, establish such a parallelism with rosefish stored at −23° C, nor could Love *(6)* with cod stored at −30° C. In both these species marked increases in toughness of texture occurred before any appreciable fall in salt extractability had taken place.

Although the workers previously referred to do not appear to have subjected their data to statistical analysis, it is possible that the differences between some of their results and those quoted here for the higher storage temperatures are due to essential differences between species. While the results suggest that texture and salt-extractability of the proteins of morwong held at temperatures as low as −18° C are directly related, it is possible that

this relationship is merely fortuitous. It is clear from the results of these other workers that salt-extractability of proteins cannot invariably be used as an index of deterioration in texture of frozen fish held at temperatures below −20° C.

The regressions in Figure 1 indicate that loss of salt-extractability in the muscle proteins occurred in two phases. During the first phase, up to one month's storage, steep falls were recorded at both temperatures with a significantly greater loss at the higher temperature. In the second phase, extending from one to 6 months the rate of loss was markedly reduced at both temperatures and was apparently independent of temperature. Similar results have been found by one of us for mullet *(Mugil dobula)*, a fatty fish, when stored at −10° C (W.A.M., unpublished data).

Although it appeared that oxygen removal from the storage atmosphere retarded the loss in salt-extractability the average differences for this property as well as for textures failed to reach significance for the vacuum as compared with the non-vacuum samples at −18° C. Stansby *(10)* found that some, but not all, species of fish retained their texture better with this method of packing.

SUMMARY

Salt-extractability of the protein of frozen fish muscle has been studied in relation to its toughness as assessed by a panel of tasters, using the method of paired comparisons. The fish were stored at −10° C and −18° C in heat-sealed M.S.A.T. cellophane bags and at −18° C packed in cans under a vacuum of 22 in Hg.

For the three storage conditions, differences in salt-extractability were associated with texture differences for storage times of two to six months. At the end of one month's storage, tasters were unable to distinguish differences in samples.

Two well-defined rates of loss of extractability were indicated by the regression of protein on time of storage. The initial rapid rate of change depended on temperature, while the subsequent slower rate was the same for the three storage conditions.

Fish stored in evacuated cans were not significantly different from the controls in texture or salt-extractable protein.

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THE EFFECT OF HEAT AND IRRADIATION ON THE MICROFLORA OF CANNED HAMS[a]

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The application of ionizing radiation to the preservation of a wide variety of food products is being investigated in many laboratories *(4, 5)*. Previous reports from this laboratory have considered the effects of irradiation on the spoilage microflora of fresh beef *(7)* and packaged frankfurters *(2)*. From the microbiological standpoint these represented two divergent types of flora that are commonly encountered in meat products. The present report is concerned with similar studies on the microflora of semi-perishable canned hams that microbiologically may be considered a third type of product.

EXPERIMENTAL METHODS

For the purpose of determining the radiation resistance of the indigenous microflora prior to heating, 4 hams that had been cured, boned, and canned, but not heat-processed, were purchased from each of 4 packing companies. The 4 companies are coded A, B, C, and D. Two of the hams from each company were opened immediately and samples removed for bacteriological analysis. The remaining 2 hams from each company were frozen at −34° C and irradiated with approximately 1,000,000 rads (1 megarad). Two of the irradiated hams were opened immediately, 2 were incubated at 25° C, and 4 were incubated at 37° C before opening for bacteriological examination. Incubated hams were examined whenever swelling of the can became obvious, or at the end of one month if the cans appeared sound. The exact treatment of each of these 16 hams is summarized in Table 1.

All irradiations of the canned hams were carried out at the Argonne National Laboratory in the M-2 container, a gamma ray source from spent fuel rods. The hams were at a temperature of approximately −30° C when placed in the irradiation cell and were irradiated at ambient temperature. Even after the one megarad irradiation (approximately 24 hr) the hams were cold to the touch. For these hams the radiation dosage was measured at various points on the surface of the can. Other experiments indicated that the decrease in dosage between the surface and center of the can ranged between 7.5 and 16% depending upon the size of the can and position in the irradiation container, with an average decrease of 11%. The core dosage given in Table 1 is based on a calculated 11% decrease from the average surface dosage.

In another experiment 16 heat-processed canned hams were purchased from the same 4 companies. Those from each company had been heat-processed in the same lot and were part of the regular production.

The precise heating schedule followed in the processing of these hams could not be obtained although it is known that each of them were processed to a core temperature of at least 150° F, the minimum required by the Meat Inspection Division.

The subsequent treatment of each of these hams is summarized in Table 2. Two cans from each company were incubated, one at 25° C, and the other at 37° C. The

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TABLE 1

Irradiation and incubation schedule for 16 raw (unprocessed) hams prior to bacteriological examination

Company and can size	Code	Irradiation dosage in megarads		Incubation	
		Surface	Center	Days	Temperature
A	A-1	0	0	0	.....
	A-2	0	0	0	.....
6¾ lb	A-3	1.07	0.95	0	.....
	A-4	1.09	0.97	33	25° C
B	B-1	0	0	0	.....
	B-2	0	0	0	.....
6¾ lb	B-3	0.98	0.87	0	.....
	B-4	1.06	0.94	33	25° C
C	C-1	0	0	0	.....
	C-2	0	0	0	.....
5 lb	C-3	1.07	0.95	30	37° C
	C-4	1.09	0.97	30	37° C
D	D-1	0	0	0	.....
	D-2	0	0	0	.....
13½ lb	D-3	0.94	0.84	30	37° C
	D-4	0.97	0.86	12	37° C

other two sets were frozen at −34° C, and irradiated, one set with approximately 0.5 megarad and the other with approximately 0.25 megarad. All irradiated, heat-processed hams were incubated at 37° C until they were swollen, or for one month if the can appeared sound.

Bacteriological examination consisted of emulsifying 10-g samples in 90 ml of sterile distilled water and quantitatively plating on APT agar *(3)*. The plates were

TABLE 2

Irradiation and incubation schedule for 16 heat processed canned hams prior to bacteriological examination

Company and can size	Code	Irradiation dosage in megarads		Incubation	
		Surface	Center	Days	Temperature
A	A-5	0	0	30	25° C
	A-6	0	0	30	37° C
6¾ lb	A-7	.49	.44	32	37° C
	A-8	.25	.23	34	37° C
B	B-5	0	0	12	25° C
	B-6	0	0	5	37° C
6¾ lb	B-7	.50	.44	11	37° C
	B-8	.30	.25	6	37° C
C	C-5	0	0	31	25° C
	C-6	0	0	27	37° C
5 lb	C-7	.50	.44	32	37° C
	C-8	.21	.19	34	37° C
D	D-5	0	0	33	25° C
	D-6	0	0	33	37° C
9¾ lb	D-7	.47	.42	32	37° C
	D-8	.30	.26	34	37° C

TABLE 3

Bacteriological examination of raw, non-irradiated canned hams prior to incubation

Ham number	Surface sample		Boned area	
	Bacteria/g	Flora[1]	Bacteria/g	Flora[1]
A-1	71×10^6	6 Microbacteria 3 Micrococci 1 Lactobacillus (−)	24×10^8	4 Microbacteria 3 Bacterium sp. 2 Micrococci 1 Gram-negative rod
A-2	47×10^4	3 Microbacteria 3 Micrococci 3 Bacterium sp. 1 Lactobacillus (−)	51×10^3	3 Micrococci 3 Lactobacilli (−) 2 Microbacteria 2 Gram-negative rods
B-1	16×10^6	9 Lactobacilli (−) 1 Leuconostoc	19×10^6	10 Lactobacilli (−)
B-2	14×10^6	10 Lactobacilli (−)	44×10^6	9 Lactobacilli (−) 1 Leuconostoc
C-1	21×10^5	6 Micrococci 4 Bacterium sp.	95×10^4	5 Micrococci 4 Bacterium sp. 1 Lactobacillus (−)
C-2	47×10^6	5 Micrococci 5 Bacterium sp.	67×10^4	5 Micrococci 4 Bacterium sp 1 Achromobacter
D-1	16×10^7	6 Enterococci 3 Lactobacilli (+) 1 Leuconostoc	12×10^6	4 Lactobacilli (−) 3 Lactobacilli (+) 2 Gram-negative rods 1 Enterococcus
D-2	67×10^7	5 Enterococci 3 Lactobacilli (−) 2 Gram-negative rods	58×10^6	3 Lactobacilli (−) 2 Lactobacilli (+) 2 Micrococci 2 Gram-negative rods 1 Enterococcus

[1] Numbers preceding names of microorganisms indicate relative proportion of total population as determined by numbers of pure cultures isolated and identified. *Lactobacillus* (−) = homofermentative; (+) = heterofermentative.

incubated for 3 days at 30° C and counted. Ten representative colonies were picked from the appropriate dilution of each sample showing a significant bacterial population, and these isolates were identified as far as the genus. The following screening tests were run on each culture: (1) Gram stain, (2) catalase production, (3) relation to oxygen, (4) motility, (5) nitrate reduction, and (6) CO_2 production from glucose. After the results of these screening tests were available, other appropriate physiological tests were conducted to complete the identification as to genus, and in many cases to give a reasonable identification as to species.

In addition, Gram strains were made from the 1:10 dilution in the Waring blender cup. In those cases where there was evidence that clostridia were present in significant numbers, aliquots were added to thioglycollate broth.

Radiation resistance studies on selected pure cultures were conducted as described previously *(7)* using washed cells or spores resuspended in TY broth (1.0% tryptone + 0.5% yeast extract). The suspensions were removed from the cobalt furnace to an ice bath after specified periods of irradiation and quantitatively plated in a suitable medium. The radiation resistance was usually expressed as an LD_{99}, the dose in rads to destroy 99% of the initial cell suspension. In most instances, irradiation was continued until the original viable population was reduced through at least 4 log cycles, and the shape of the survivor curve was determined.

TABLE 4

Bacteriological examination of raw, irradiated canned hams after incubation[1]

Ham number	Incubation	Condition of ham	Bacteria/g	Flora
A-3	none	No gas, off-color or odor	<10	.....
A-4	25° C; 33 days	No gas or off-color, slight off-odor	<10	.....
B-3	none	No gas or off-color, slight off-odor	<10	.....
B-4	25° C; 33 days	Slight gas, no off-color, slight off-odor	<10	.....
C-3	37° C; 30 days	No gas or off-color, slight off-odor	<10	.....
C-4	37° C; 30 days	No gas or off-color, slight off-odor	<10	.....
D-3	37° C; 30 days	No gas or off-color, slight off-odor	<10	.....
D-4	37° C; 12 days	Gas, no off-color or odor	2×10^8	10 Enterococci

[1] All of these hams received approximately one megarad irradiation. Precise doses are given in Table 1.

TABLE 5

Bacteriological examination of heat processed, non-irradiated canned hams after incubation

Ham number	Incubation	Condition of ham	Bacteria/g	Flora[1]
A-5	25° C; 30 days	No gas, off-color or odor	<100	.....
A-6	37° C; 30 days	No gas, off-color or odor	67×10^7	10 Enterococci
B-5	25° C; 12 days	Hard swell, putrid odor	69×10^6	10 Bacilli
B-6	37° C; 5 days	Hard swell, putrid odor	25×10^6	10 Bacilli
C-5	25° C; 31 days	No gas. Off-odor, gelatin hydrolyzed and turbid	76×10^7	7 Enterococci 3 Lactobacilli
C-6	37° C; 27 days	Slight gas, off odor, gelatin hydrolyzed and turbid	51×10^7	10 Enterococci
D-5	25° C; 33 days	No gas, off-color or odor	<100	.....
D-6	37° C; 33 days	Slight gas, no off-color or odor	240×10^4	10 Bacilli

[1] Numbers preceding names of microorganisms indicate relative proportion of total population as determined by numbers of pure cultures isolated and identified.

TABLE 6

Bacteriological examination of heat processed, irradiated canned hams after incubation

Ham number	Radiation dosage on surface (megarads)	Incubation (days at 37° C)	Condition of ham	Bacteria/g	Flora[1]
A-7	.49	32	No gas, off-color or odor	<10	.....
A-8	.25	34	No gas, off-color or odor	<10	.....
B-7	.50	11	Hard swell, putrid, gelatin hydrolyzed, mushy	aerobic-12×10^8; anaerobic-12×10^6	10 Bacilli 10 Clostridia
B-8	.30	6	Hard swell, putrid, gelatin hydrolyzed, mushy	aerobic-20×10^4; anaerobic-20×10^7	10 Bacilli 10 Clostridia
C-7	.50	32	No gas, off-color or odor	<10	.....
C-8	.21	34	No gas, off-color or odor, gelatin turbid	78×10^3	10 Lactobacilli (+)
D-7	.47	32	No gas, off-color or odor	30×10^6	10 Bacilli
D-8	.30	34	No gas, off-color or odor	122×10^8	10 Bacilli

[1] Numbers preceding names of microorganisms indicate relative proportion of total population as determined by numbers of pure cultures isolated and identified.

Heat resistance studies were carried out by heating, in a constant temperature water bath, cells freshly suspended in a suitable growth medium. After the desired time intervals, tubes were removed from the water bath to an ice water bath and then placed in an incubator.

RESULTS

Microflora of raw and heat processed canned hams before and after irradiation. Results of the bacteriological examination of the raw, non-irradiated hams are summarized in Table 3. The total count and type of flora were very similar between each pair of hams from the same company. In general, the surface count was higher than the count from the inner surfaces adjacent to the boned area, but the type of flora in these two locations was similar. Hams from companies B and D had relatively high bacterial counts, and most of the bacteria were homofermentative lactic acid bacteria that are very difficult to differentiate morphologically as lactobacilli or streptococci. Hams from company A had the lowest count with microbacteria being the most common, but with some micrococci and Gram-positive rods detected. The hams from company C had an intermediate count, about evenly divided between micrococci and Gram-positive rods. These rods are identified as *Bacterium sp.* because no spores could be detected. However, it is possible that they actually are *Bacillus* strains. Most of the micrococci tended to form tetrads in broth culture and may be related to the genus *Gaffkya*.

The results with the 8 raw, irradiated hams are summarized in Table 4. Only one of these hams showed bacterial growth after approximately one megarad irradiation and 30-day incubation. This ham contained 2×10^3 enterococci per gram.

The results of the 8 heat processed, non-irradiated hams are summarized in Table 5

and the 8 heat processed, irradiated hams in Table 6. Two of the heat processed hams showed no growth after incubation, 3 contained bacilli, and 3 contained enterococci. Lactobacilli also were found in one of the hams containing streptococci.

Three of the heat processed irradiated hams showed no bacterial growth after irradiation and incubation. However, one contained homofermentative lactobacilli, and 4 had moderate numbers of bacilli. The anaerobic spore-forming bacilli which predominated in hams B-7 and B-8 were not isolated or identified other than by morphological and cultural characteristics.

Taxonomic study of Bacillus strains from canned hams. Thirteen *Bacillus* cultures isolated from the canned hams in the present study, and 24 *Bacillus* cultures that had been isolated by the laboratories of 4 meat packing companies from spoiled commercial canned hams were identified as to species using the methods suggested by Smith *et al (6)*. The source of these cultures and their identification are summarized in Table 7.

Nineteen of the 37 strains were identified as *Bacillus licheniformis*. The cultures from the 4 heat processed hams from company B and 15 of the strains received from other laboratories were members of this species. Some of the distinctive characteristics noted among these cultures, especially those of importance in relation to their growth in canned hams are as follows: Relatively small size with central cylindrical or oval

TABLE 7

Identification of Bacillus species isolated from heat processed canned hams

Source	Ham number	Number of strains	Identification
Company B	B-5	1	*B. licheniformis*
	B-6	1	*B. licheniformis*
	B-7	1	*B. licheniformis*
	B-8	1	*B. licheniformis*
Company D	D-6	3	*B. coagulans*
	D-7	3	*B. coagulans*
	D-8	3	*B. coagulans*
Received from Company 1	.....	1	*B. licheniformis*
Received from Company 2	.....	1	*B. cereus*
		1	*B. subtilis*
Received from Company 3	.....	13	*B. licheniformis*
		4	*B. subtilis*
		2	*B. cereus*
		1	*B. coagulans*
Received from Company 4	.....	1	*B. licheniformis*

spores which do not swell the sporangia; ability to grow anaerobically; gas production from nitrates anaerobically, and to some extent from glucose when cultured anaerobically; final pH of 5.2 or less in glucose broth cultured anaerobically; gelatin, casein, and starch rapidly hydrolyzed; prompt growth in 7.5% NaCl broth, and some growth by most strains in 15% NaCl. Two strains included in this species were identical to it in all respects except that spores could not be demonstrated. Although a number of different media and techniques were attempted for this purpose, no spores have been found in these 2 strains as yet.

The isolates from 3 of the heat processed hams from company D and 1 of the strains received have been identified as *Bacillus coagulans*. These cultures were relatively small rods with terminal spores and swollen sporangia. They grew well anaerobically but produced no gas from glucose or nitrate. The final pH in glucose broth aerobically and anaerobically was below 5.2. Starch was hydrolyzed, but gelatin and casein were hydrolyzed very weakly or not at all. These cultures did not grow in a broth containing 5% NaCl. These cultures grew well at 37° C but little, or not at all, at 30° C.

Five strains received from other laboratories were identified as *Bacillus subtilis*. These were small rods with central spores which produced no swelling of the sporangia. Anaerobically, they grew very poorly, if at all. Gelatin, casein, and starch were hydrolyzed. All of these strains grew well in 7.5% NaCl broth and showed some growth in 12.5% NaCl. One strain was black pigmented when received, but when re-isolated, it did not blacken carbohydrate or tyrosine media. Evidently, a stable dissociant of either *B. subtilis* var. *niger* or *B. subtilis* var. *aterrimus* was inadvertently isolated.

Three of the strains received were identified as *Bacillus cereus*. These were large rods with central oval to cylindrical spores that produced no swelling of the sporangia. On media containing glucose, many large vacuoles were observed in the cells. All grew well anaerobically and 2 produced gas from nitrate anaerobically. Gelatin, casein, and starch were hydrolyzed. They produced acetoin from glucose, and no acid from mannitol.

Radiation resistance of Bacillus spores. Spore suspensions of 3 representative *Bacillus* strains were prepared and subjected to irradiation in the Cobalt-60 furnace. The LD_{∞} of the *B. licheniformis* strain was 249,000 rads, the *B. coagulans* strain 338,000 rads, and the *B. cereus* strain 513,000 rads.

Heat resistance of lactic acid bacteria from canned hams. Ten representative strains of lactic acid bacteria that were isolated from the canned hams in the present study were further identified as to species, and their heat resistance was determined. These results are summarized in Table 8. Two strains of streptococci differed in

TABLE 8

Heat and radiation resistance of lactic acid bacteria from canned hams

Species	Source (Ham number)	Min at 145° F		LD_{∞} (kilorads)
		Survived	Killed	
Streptococcus durans	C-6	125	130	
Streptococcus faecium	C-5	100	105	
Streptococcus faecalis	A-6	80	85	
Streptococcus faecalis	A-6	55	60	
Streptococcus faecalis var. *liquefaciens*	C-5	35	40	
Enterococcus sp.	D-1	3	6	
Enterococcus sp.	D-4	3	6	160
Lactobacillus plantarum	C-1	3	6	
Lactobacillus plantarum	C-5	0	3	
Lactobacillus plantarum	C-8	0	3	330

several respects from known species and are listed as *Enterococcus sp.* The *Streptococcus durans* and *Streptococcus faecium* strains were found to be particularly resistant. This is in accord with our previous study of the heat resistance of a rather large collection of enterococci *(1)* in which it was noted that *S. faecium* strains from spoiled commercial canned hams were particularly heat resistant.

Radiation resistance of lactic acid bacteria from canned hms. The radiation resistance of the unidentifiable enterococcus from ham D-4, that had received approximately one megarad, and a *Lactobacillus plantarum* from ham C-8, that had received approximately 0.2 megarad, is also given in Table 8.

Heat and radiation resistance of enterococci from canned hams and other sources. Table 9 records the heat and radiation resistance of several strains of enterococci from canned hams and other sources. Particularly noteworthy is strain HS-5 of *S. faecium*. This strain survived 130 min at 145° F and as much as 55 min at 150° F, and its radiation resistance was as high as that of most bacterial spores. It also may be noted that there is no direct correlation between heat resistance and radiation resistance. In addition, it was observed that strains of enterococci from canned hams generally were considerably more heat resistant than those from fecal sources.

Strain HS-5 of *S. faecium* was selected to study the effect of combined heat and irradiation. When survivors of a heat treatment that killed 90% of the cells were subjected to irradiation, the survivor curve had lost some of its initial lag or induction

TABLE 9

Heat and radiation resistance of enterococci from canned hams and other sources

Species	Strain	Source	Min at 145° F		LD_{99} (kilorads)
			Survived	Killed	
Streptococcus faecium	HS-5	canned ham	130	140	420
Streptococcus faecium	HD-4B	canned ham	100	110	280
Streptococcus faecium	HF-2A	canned ham	80	90	170
Streptococcus faecium	R-53	canned ham	70	80	650
Streptococcus faecium	US-7	pig gut	60	70	360
Streptococcus faecium	ARA-10	pig gut	20	30	240
Streptococcus durans	6056	ATCC	90	100	160
Streptococcus durans	8043	ATCC	16	20	380
Streptococcus durans	ARA-2	pig gut	16	20	300
Streptococcus faecalis	3 GAP-2	human feces	40	50	140
Streptococcus faecalis	5 GAP-2	human feces	30	40	140
Streptococcus faecalis var. *liquefaciens*	ARD-1	pig gut	15	20	140

phase, but the curve was generally parallel to that obtained with the unheated cells. Cell suspensions that were initially irradiated to kill 90% of the cells appeared to be slightly more sensitive to heat than were unirradiated cells. However, the results were not striking and the main change in the survivor curve again was elimination of the initial lag phase. The presence of 5% NaCl in the suspending medium also appeared to sensitize the cells to both heat and irradiation.

DISCUSSION

These studies indicate that the bacterial load in canned hams prior to heat processing generally is unaccountably high and is composed largely of a variety of lactic acid bacteria. Irradiation with one megarad of gamma rays is adequate to destroy most of these organisms, but adversely affects the odor and flavor of the product.

The usual commercial heat processing is not designed to kill the spores of clostridia and bacilli, and occasionally some of the lactic acid bacteria also survive. During subsequent refrigerated storage, surviving lactic acid bacteria may grow extensively and produce a sour product without producing any gas in the can. In the present study, of the 8 canned heat processed hams incubated at warm temperatures, only 4 showed any evidence of gas production, and 2 of the sound cans showed no evidence of growth of surviving organisms after one month at 25° C. Irradiation of the heat processed hams at 0.2 to 0.5 megarad had only a slight additional effect on the stability of incubated hams. Two of the four hams that received 0.5 megarad were sound after one month's incubation and the other two were spoiled by sporeforming bacteria.

The present study indicates that any combination of heat and irradiation that is adequate to sterilize canned hams will be of a severity that will adversely affect the acceptability of the product. Furthermore, the enterococci (particularly *S. faecium*) that sometimes survive otherwise adequately heat-processed hams also are unusually resistant to irradiation. Thus, low level irradiation would not be expected to reduce significantly the amount of heat processing required to produce a ham that is stable when refrigerated.

SUMMARY

The bacterial flora of canned hams before heat processing consisted largely of a variety of lactic acid bacteria. Either the normal commercial heat processing or irradiation with approximately one megarad of gamma irradiation destroyed most of the flora but permitted survival of spores of bacilli and clostridia and occasionally non-sporeforming lactic acid bacteria. The most troublesome of these latter organisms would appear to be *Streptococcus faecium*, some strains of which were unusually resistant to both heat and irradiation. The combination of moderate levels of heat and irradiation did not appear to have any particular advantage over present methods of heat processing followed by refrigerated storage.

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STUDIES ON THE INACTIVATION OF TYPE A *CLOSTRIDIUM BOTULINUM* TOXIN BY IRRADIATION WITH COBALT 60[a, b]

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Spores of *Clostridium botulinum* are heat resistant. Any of these spores remaining in a non-acid medium stored under anaerobic conditions may grow and produce toxin. This toxin is heat labile; therefore, heating to boiling will inactivate it. The fact that *Cl. botulinum* toxin is readily destroyed by heating suggested that irradiation may provide an alternative method of easily inactivating botulinum toxin.

Unpurified *Cl. botulinum* toxin in both broth and surface ripened cheese media was irradiated with a Cobalt-60 source, and extremely high dosages of irradiation were required for inactivation. Experiments were designed to determine: (a) the irradiation dosage required to reduce the toxicity of cheese or broth samples containing varying i al toxin levels to the minimum level measurable by intraperitoneal mouse injections (in this instance to less than 20 MLD per g of sample) and (b) the general pattern of reduction in toxicity as progressively higher doses of irradiation were applied to similar toxic samples.

In sodium phosphate buffer solution purified type A botulinum toxin was readily inactivated by irradiation *(3)*. The question then was whether or not purified toxin in the presence of food or broth medium would be similarly affected by irradiation. Purified type A botulinum toxin remaining from the latter experiments was used in a series of three tests by adding it to sterile trypticase broth and heated surface ripened cheese before irradiation with Cobalt-60.

MATERIALS AND METHODS

The following strains of *Cl. botulinum* were used to produce crude toxin:

Strain	Type	Obtained from
T	A	Isolated in laboratory of Dept. of Microbiology, Univ. of Chicago, from an unpurified culture from cheese spread, received from the Food Research Laboratories, New York, N. Y.
109	A	Dr. K. F. Meyer, Hooper Foundation, Univ. of California.

Crude *Cl. botulinum* toxin was produced by inoculating 100 ml portions of beef heart medium in 250 ml Erlenmeyer flasks with culture and incubating at 30° C for 10 days. The toxic broths were freed of *Cl. botulinum* spores by centrifugation and filtration through a Seitz filter. After titration in mice for toxicity, the toxin was

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[b] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 27, 1958.

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diluted to the desired concentration with sterile trypticase broth or added to heat treated surface ripened cheese.

Surface ripened cheese was warmed in a water bath at 70° C and stirred until homogeneous prior to the addition of the crude toxin. The cheese was weighed into a beaker, pasteurized at 90° C for 10 min and cooled to below 50° C. Sterile distilled water was added to restore the moisture lost during heating. Sufficient toxic broth (usually 10 to 15%) was added to yield the desired toxin level in the cheese. Four g portions were weighed into sterile 15 x 125 mm culture tubes. Four ml aliquots of diluted toxic broth were pipetted into similar tubes. The air above the toxic broth or cheese was evacuated to 400 mm Hg. In tubes containing cheese the air was replaced with a mixture of 90% N_2 and 10% CO_2. The tubes were constricted approximately one inch above the surface of the sample and sealed. These samples were stored in a refrigerator until needed for irradiation.

The toxin in the cheese and broth samples was assayed at 3 levels; namely, 1,000, 200 and 20 MLD by duplicate intraperitoneal injections into 16 to 20 g white mice of 0.5 ml amounts of appropriate dilutions. The lowest level detected in cheese was 20 MLD without encountering non-specific deaths from breakdown products. Similar titration levels were used for the broth samples for analytical comparisons.

In experiments in which buffer solution, broth and cheese were to be compared as suspending media, purified toxin was added to sterile trypticase broth and heat treated surface ripened cheese. These broth and cheese preparations were tubed as described earlier, using an atmosphere of 90% N_2 and 10% CO_2 above all samples. Each day that irradiations were made, dilutions of toxin calculated to have the same concentration as those in the broth and cheese were prepared in 0.05*M* sodium phosphate buffer solution from the concentrated toxin. Four ml portions were dispensed to tubes and sealed like the broth and cheese.

Tubes of buffer solution, broth and cheese were irradiated with Cobalt-60 at several levels to establish each inactivation pattern. The temperature of irradiation varied between 3.5° and 8.0° C. After each irradiation exposure the tubes were opened, appropriate dilutions made in gelatin-phosphate diluent *(1)* and 16 to 20 g white mice injected intraperitoneally in 0.5 ml amounts. For the irradiated samples 4 mice were inoculated with each of several dilutions. For unirradiated samples 3 mice were inoculated with each dilution. Two or more separate series of inoculations were used to establish the toxicity of the unirradiated specimens. The mice were observed 4 days.

RESULTS

Effect of irradiating toxin of Cl. botulinum in surface ripened cheese. Preliminary experiments designed to test the effect of gamma irradiation on *Cl. botulinum* toxin inoculated into surface ripened cheese showed that higher levels of irradiation than anticipated were required to inactivate botulinum toxin in this medium. Therefore, large irradiation dosages were used on surface ripened cheese inoculated with type A *Cl. botulinum* toxin produced in beef heart medium (Table 1). In one trial with toxin produced by strain T, the dosage required to reduce the toxicity from above 1,000 MLD to below 20 MLD was 7,300,000 rads. Similar results were observed in three trials with toxin produced by strain 109. In Trial 1 the toxicity was not reduced from above 1,000 MLD to below 20 MLD by 7,300,000 rads. In Trial 2 this dosage was sufficient to reduce a comparable level of initial toxicity to less than 20 MLD. In Trial 3 a toxicity above 1,000 MLD was not reduced to below 20 MLD by 6,700,000 rads. These levels of irradiation were much higher than those required to inactivate *Cl. botulinum* spores in surface ripened cheese, and slightly higher than the dosage required to reduce similar levels of type A *Cl. botulinum* toxin in broth to a comparable end point.

Effect of irradiating toxin of Cl. botulinum in trypticase broth. After observing the slow inactivation of *Cl. botulinum* toxin in cheese by irradiation with Cobalt-60, it was suspected that this medium possibly contributed protection to the toxin. Therefore, several experiments were conducted in which unpurified type A *Cl. botulinum* toxin in trypticase broth was irradiated at varying dosages. Data from 4 experiments with toxic trypticase broths are shown in Table 2.

TABLE 1

The effect of varying dosages of irradiation with Cobalt-60 on type A Clostridium botulinum toxin in surface ripened cheese in partially filled sealed tubes[1]

Irradiation dosage (rads) (000 omitted)	Toxin produced by			
	Strain T	Strain 109		
	Trial 1 MLD toxin	Trial 1 MLD toxin	Trial 2 MLD toxin	Trial 3 MLD toxin
None	1,000[2]	1,000	1,000	1,000
330	1,000	1,000	1,000	1,000
490	1,000	1,000	1,000	1,000
650	200	1,000	1,000	1,000
800				1,000
900	200	1,000	1,000	200
1,300	200	200	200	200
1,600	200	200	200	200
2,000	200	200	200	200
2,900	200	200	200	20
3,900	20	200	200	20
4,800	20	20	20	20
5,900	20	20	20	20
6,700				20
7,300	Less than 20	20	Less than 20	

[1] Atmosphere above cheese in tubes was 90% N_2 + 10% CO_2.
[2] Between 1,000 and 10,000 MLD.

In Trial 1 with toxic broth produced by strain T, 4,000,000 rads was insufficient to reduce the toxicity from above 1,000 MLD to below 20 MLD. In the second trial a toxic broth from strain T, which had an initial toxicity near 1,000 MLD was reduced by 4,000,000 rads to below 20 MLD. The toxic broths produced by strain 109 had higher initial toxin levels. In Trial 1 with toxic broth from this source, the toxin level was not reduced from above 1,000 MLD to below 20 MLD by 4,000,000 rads. The second broth sample from strain 109 had an even higher initial toxin level that was not reduced to below 20 MLD by 4,900,000 rads.

TABLE 2

The effect of varying dosages of irradiation with Cobalt-60 on type A Clostridium botulinum toxin in trypticase broth in partially filled sealed tubes[1]

Irradiation dosage (rads) (000 omitted)	Toxin produced by			
	Strain T		Strain 109	
	Trial 1 MLD toxin	Trial 2 MLD toxin	Trial 1 MLD toxin	Trial 2 MLD toxin
None	1,000[2]	1,000	1,000	1,000
330	1,000	200	1,000	1,000
500	200	200	1,000	1,000
670	200	200	1,000	1,000
1,000	200	200	200	1,000
1,300	200	200	200	1,000
1,700	200	200	200	1,000
2,000	200	20	200	1,000
2,500	20		200	
3,000	20	20	20	200
3,500	20		20	
4,000	20	Less than 20	20	20
4,900		Less than 20		20

[1] Atmosphere above broth in tubes was air.
[2] Between 1,000 and 10,000 MLD.

In cheese and broth containing type A *Cl. botulinum* toxin there was an orderly destruction of the toxicity during the extended irradiation exposure required to reduce the varying initial amounts of toxin to the proposed endpoint (less than 20 MLD).

Effect of irradiating purified Cl. botulinum toxin in buffer solution, broth and cheese. Data from 3 experiments in which comparative studies were conducted on the irradiation inactivation of purified type A *Cl. botulinum* toxin in 0.05*M* sodium phosphate buffer solution, 5% trypticase broth and surface ripened cheese are shown in Tables 3, 4 and 5. A rapid reduction in the toxicity of the purified toxin in pH 7.5 sodium

TABLE 3

A comparison of the effect of irradiation with Cobalt-60 on purified type A Clostridium botulinum toxin in sodium phosphate buffer solution, trypticase broth, and heated surface ripened cheese[1]

Irradiation dosage (rads) (000 omitted)	Sodium phosphate buffer solution (0.05M—pH 7.5) MLD toxin	Trypticase broth (5% solution) (pH 7.1) MLD toxin	Surface ripened cheese (58% solids) (pH 6.2) MLD toxin
None	20,000	20,000	20,000
6	2,000		
58	50		
180	20		
340	10		
700	Less than 5		
1,000	Less than 5		
1,400	Less than 5		
2,800		2,000	10,000
5,600		500	2,000
8,400		200	1,000
11,000		20	200
14,000		10	100
20,000		Less than 5	20

[1] Atmosphere above samples in tubes was 90% N_2 + 10% CO_2.

phosphate buffer and a decreased rate of reduction of the same toxin occurred in either trypticase broth or cheese. For example, in the first experiment (Table 3) the toxic buffer solution was reduced from 20,000 MLD to less than 5 MLD by 700,000 rads. The same initial concentration of toxin in broth required 20,000,000 rads for reduction to less than 5 MLD. In cheese 20 MLD of toxicity remained after irradiation with 20,000,000 rads. The lowest level of toxin that can be tested in cheese is 20 MLD without possibly encountering non-specific deaths from the diluted cheese medium. Similar inactivation patterns were observed in the second and third experiments (Tables 4 and 5) where the initial toxin concentration was 200 MLD rather than 20,000 MLD. These data indicated that the complex media (5% broth or cheese) contributed protection to the toxin which increased the inactivation dosages approximately one hundredfold above those required to inactivate the toxin pH 7.5 sodium phosphate buffer solution. The differences between the inactivation dosages for broth and cheese were usually less than twofold, which indicated that the protective effect of 5% solids is almost as great as 50% solids. There probably was adequate protein material in the broth to shield the toxin molecules because the inactivation patterns were similar whether the initial concentration of toxin was 20,000 or 200 MLD. Other components of the cheese (e.g. butterfat) may have contributed to the greater protective effect of cheese, although the higher protein concentration also may have been an important factor.

DISCUSSION

The data on broth and cheese to which purified type A *Cl. botulinum* toxin was added are in agreement with earlier experiments in which type A *Cl. botulinum* toxin was produced in broth culture and subsequently

TABLE 4

A comparison of the effect of irradiation with Cobalt-60 on purified type A Clostridium botulinum toxin in sodium phosphate buffer solution, trypticase broth, and heated surface ripened cheese[1]

Irradiation dosage (rads) (000 omitted)	Sodium phosphate buffer solution (0.05M—pH 7.5) MLD toxin	Trypticase broth (5% solution) (pH 7.0) MLD toxin	Surface ripened cheese (57% solids) (pH 6.9) MLD toxin
None	200	200	200
3	100		
6	100		
14	20		
29	10		
57	Less than 5		
110	Less than 5		
170	Less than 5		
340	Less than 5		
510	Less than 5		
680		200	200
1,400		100	200
2,700		50	100
5,500		10	50
8,200		Less than 5	Less than 20

[1] Atmosphere above samples in tubes was 90% N_2 + 10% CO_2.

freed of spores by Seitz filtration. For example, with the purified toxin in cheese (Table 4) 8,200,000 rads were required to reduce the toxicity from 200 MLD to less than 20 MLD. Similarly, 5,500,000 rads were required to reduce the toxicity of trypticase broth from 200 MLD to 10 MLD. When unpurified *Cl. botulinum* toxin was added to cheese and broth, cheese was reduced from a toxicity of 1,000 MLD to less than 20 MLD by 7,300,000 rads (Table 1) and broth from a toxicity of 1,000 MLD to less than 20 MLD by 4,000,000 rads (Table 2).

TABLE 5

A comparison of the effect of irradiation with Cobalt-60 on purified type A Clostridium botulinum toxin in sodium phosphate buffer solution, trypticase broth, and heated surface ripened cheese[1]

Irradiation dosage (rads) (000 omitted)	Sodium phosphate buffer solution (0.05M—pH 7.5) MLD toxin	Trypticase broth (5% solution) (pH 7.0) MLD toxin	Surface ripened cheese (59% solids) (pH 6.0) MLD toxin
None	200	200	200
3	100		
6	50		
14	20		
28	5		
57	Less than 5		
680		200	200
1,400		100	200
2,700		50	100
5,400		5	20
8,100		Less than 5	20
11,000			20

[1] Atmosphere above samples in tubes was 90% N_2 + 10% CO_2.

When CO_2, 90% N_2 + 10% CO_2, and air were compared as atmospheres above toxic trypticase broth in partially filled sealed tubes, there was no apparent differential influence on the rate of inactivation of the *Cl. botulinum* toxin in the broth *(2)*.

Cl. botulinum toxin is labile to heat when in solution or in a medium containing a high percentage of solids. Although botulinum toxin is readily inactivated by irradiation when in a dilute buffer solution, its tolerance to inactivation increases when additional protein is present; therefore, irradiation is not a satisfactory alternative method of inactivating *Cl. botulinum* toxin.

SUMMARY

Experiments designed to test the effect of gamma irradiation with Cobalt-60 on *Clostridium botulinum* toxin added to surface ripened cheese disclosed that levels of irradiation higher than anticipated were required to inactivate botulinum toxin in this medium.

Unpurified type A *Cl. botulinum* toxin added to cheese required higher levels of irradiation to reduce the toxicity to below 20 MLD than toxin irradiated in a broth medium. In cheese, where the initial toxin level was above 1,000 MLD, reduction of the toxicity to below 20 MLD required at least 7,300,000 rads. In broth toxin levels above 1,000 MLD were not reduced to below 20 MLD by less than 4,000,000 rads, and mainly at least 4,900,000 rads were required.

In contrast, purified type A *Cl. botulinum* toxin in 0.05*M* sodium phosphate buffer solution was reduced from 20,000 MLD to below 20 MLD with 340,000 rads, or from 200 MLD to less than 20 MLD with only 29,000 rads.

Purified type A *Cl. botulinum* toxin was added in the same concentration to 0.05*M* sodium phosphate buffer solution (pH 7.5), sterile 5% trypticase broth and heat treated surface ripened cheese. These preparations were subjected to gamma irradiation with Cobalt-60 at several dosage levels. Three experiments showed that the addition of 5% trypticase broth to the toxin increased the irradiation dosage necessary to inactivate the toxin approximately one hundredfold compared to the irradiation of toxin in sodium phosphate buffer solution. Toxin was protected from inactivation slightly more by the cheese medium than by broth.

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RESISTANCE OF *BACILLUS COAGULANS* SPORES TO GAMMA RAYS. APPLICATION OF THE MULTIPLE TUBE PROBABILITY METHOD[a]

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In general, two methods have been commonly used to measure radiosensitivity of bacteria. One involves the determination of the minimum sterilizing dose for a definite bacterial population. This procedure permits precision in determining a point on the survival curve, but fails to define the slope of the curve. The point of destruction varies with the initial number of cells or spores. The more commonly used second method relies on estimating the fractions of microbial survivors for graduated doses. However, conventional plate counting procedures used for this purpose do not give precise values when the number of survivors is small, especially in the range below 10 per ml. The latter method does furnish satisfactory data on the slope of the survival curve if the selected plating and cultural conditions are favorable for growth. The present report describes a multiple tube probability procedure which applies advantageous features of both of the above methods and improves the reliability of data by permitting the use of large numbers of replicate samples per radiation dose with rapidity and ease.

The sensitivity of *Bacillus coagulans (B. thermoacidurans)* spores to gamma rays has been studied by several investigators. Proctor and his colleagues *(8, 9, 10, 14, 15)* found that irradiation of the spores with gamma or with cathode rays under atmospheres of air, oxygen, nitrogen or vacuum did not appear to affect their radioresistance either in frozen or in non-frozen saline. However, sensitivity was affected by pH or type of suspending medium used, and by the age of the spores irradiated. The mode of death was apparently exponential. Morgan and Reed *(13)* reported that the resistance of the spores to irradiation could be changed by the incubation temperature at which they were grown, as well as by the amount of aeration during growth. Tarpley *et al (21)*, working with mixtures of organisms including *B. coagulans* spores, concluded that radiation doses between 2.5 and 3.0 megarad were required to sterilize cortisone acetate containing initial counts of 10^8/ml.

Since *B. coagulans* is an important food spoilage organism, for which some radiosensitivity data are available, it was selected for additional study with the multiple tube probability method.

METHODS AND MATERIALS

Test organism. *Bacillus coagulans* 43P, obtained from Dr. C. F. Schmidt, Continental Can Co., Chicago, Illinois, was employed in these studies. Stock cultures of the

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organism were maintained on slants of trypticase soy agar (BBL) fortified with 0.5% yeast extract.

Preparation of standardized spore suspension. Twenty 32-oz prescription bottles, each containing 200 ml of thermoacidurans agar (Difco) as slants, were inoculated with 3 ml of a 24-hr culture of *B. coagulans* grown in thermoacidurans broth at 45° C, spread uniformly over the agar surface, and incubated for 7 days at 45° C. The cultures were washed off the slants with M/15 phosphate buffer pH 7.0, combined, and washed 4 times by alternate centrifuging and resuspension with buffer. The spore crop was suspended in 300 ml of the buffer in a 1000-ml sterile Erlenmeyer flask containing glass beads, pasteurized at 95° C for 5 min to destroy vegetative cells and possible air contaminants, and stored at 5° C. A most probable number (MPN) count was performed on the stock spore suspension by preparing consecutive decimal dilutions in buffer and subculturing one-ml aliquots in five replicate screw cap tubes of MPN medium. This medium consisted of thioglycollate medium No. 136C (BBL) which had been enriched with 0.5% glucose, 50 ppm L-alanine, 10 ppm adenosine and 10 ppm Mn^{++} as $MnSO_4$ (MPN medium). Of 21 spore germination media examined, the latter medium produced the most satisfactory results. The tubes were incubated for 6 weeks at 45° C and the number of spores estimated by employing Fisher and Yates statistical tables *(7)*.

Filling of glass tubes. A small quantity (2 to 3 ml) of the stock spore suspension was heat-activated at 95° C for 5 min, suitably diluted either in chilled phosphate buffer pH 7.0 or in protein-fortified skim milk[b] to yield approximately 1×10^6 spores per ml, and shaken vigorously by hand for 15 min to uniformly disperse the spores. Employing an automatic pipetting machine, one-ml amounts of the suspension were distributed aseptically into sterile 10 x 75 mm pyrex tubes. The dispensing technic was that of Anellis, Lubas, and Rayman *(1)*. The tubes were then stoppered with sterile cotton plugs.

Canning of tubes. A set of 10 tubes was inserted in each No 2 can containing gelled 2% agar. Holes had been bored in the agar to hold the tubes snugly at a depth such that the center of the spore suspension was located in the plane midway between the top and bottom of the can, which corresponded with the center position of the dosimeter. Because 2% agar gel is practically unit density, dosimetry comparisons between cans filled with gelled agar and with water showed no measurable differences. The tubes were spaced uniformly along a circumference about ¼-in from the can wall. To prevent the wetting of the cotton plugs due to condensate collecting at the top of the cans, it was necessary to allow 1- to 1¼-in of headspace above the agar in the cans and to place 4 layers of filter paper disks over the tubes. The cans were then sealed in the presence of air or nitrogen. Nitrogen gassing was accomplished by slowly evacuating the chamber of the can closing machine to a pressure of 0.5 atmosphere and holding at this level for 1.5 min, then filling the chamber with nitrogen to a pressure of 1.5 atmosphere and holding for 1.5 min. The evacuation-nitrogen-flushing-cycle was repeated and the cans were sealed under nitrogen gas at a pressure of 15 mm Hg above atmospheric. This treatment produced a final oxygen concentration in the headspace of the cans of about 2% by Orsat analysis. Sealed cans were packed in wet ice for 20 to 24 hr during storage and transportation to the radiation source.

Radiation treatment and dosimetry. Cans were exposed to gamma rays at the High Level Gamma Irradiation Facility of the Argonne National Laboratory. The radiation souce and irradiation treatment of samples has been described *(2)*. Irradiation sites were selected which possessed little or no vertical variation in gamma flux. To prevent excessive thermal rise during irradiation, two No 2 cans of ice were placed both above and below the test cans. Using exposure periods of 50 to 70 min, the temperature of the substrate rose only 9 to 12° C above its initial temperature (2–4° C). Measurements of the dose rate *(19)* and time of exposure were performed by Argonne personnel. Dosage was controlled by varying the time of exposure at a selected dose rate, all cans for a given experimental series being held at one specific site within the radiation source. Increments of about 100 kilorads were selected for spacing the doses. After irradiation the cans were held in wet ice overnight before opening, unless stated otherwise.

Recovery of irradiated spores. In order to detect surviving spores in irradiated samples, one ml of MPN medium was added aseptically to each tube and, after sealing

[b] 10% skim milk + 0.5% yeast extract (BBL) + 0.5% phytone (BBL).

in an oxy-gas flame, the tubes were incubated at 45° C for 6 weeks. Double-strength MPN medium was used for experiments with phosphate buffer suspensions, single-strength broth being employed with milk suspensions. The growth of the organism was excellent under these anaerobic conditions. Visible turbidity readings of the tube contents were made for phosphate buffer suspensions at weekly intervals, and the number of tubes out of 10 showing survivors at each irradiation dose level was recorded. Positive growth samples in fortified milk were detected by turbidity throughout the tubes and by clotting of the casein. It was noted that a significantly higher number of positive tubes were obtained in the milk substrate by the addition of single-strength MPN medium than in its absence. As a supplemental precaution to rule out possible contaminating organisms, whenever endpoints of survival for a set showed fewer than 4 positive tubes out of 10, those cultures were confirmed by agglutination reactions with antiserum prepared from the test organism and by a bromocresol purple acidity test.

Calculation of radiation resistance. The computations adopted are based on the assumption that the order of bacterial death by ionizing radiations is logarithmic, and is expressed in terms of D_D, the slope of the linear radiation resistance curves *(17)*. The value of D_D, the dose required to reduce a bacterial population by 90%, was calculated adapting the probability method of Schmidt *(18)*, originally developed for treating heat resistance data. The latter method is modified only by substituting the irradiation dose for the exposure time to heat.

The calculation procedure is illustrated in Table 1 using the experimental data of Table 3. Thus, in an experimental series employing 7 test doses, the survival-kill data are available from a total of 70 tubes. For any set of 10 tubes, dependent on the dose, culturing may show survivors present: (a) in all 10 tubes; (b) in none; or (c) in some but not all of the 10 tubes. Consequently, endpoints and partial-survival points are obtainable. An endpoint, therefore, is the dose range between the highest dose showing some positive cultures and the lowest dose showing all negative cultures.

TABLE 1

Calculation of D_D value from data in Table 3 according to Schmidt's probability procedure

Dose Krad	Number of tubes			m	n	m + n	P
	Tested	+	−				
740	10	10	0	22	0	22	0.04
840	10	8	2	12	2	14	0.19
930	10	4	6	4	8	12	0.64
1020	10	0	10	0	18	18	0.95

$$D_D = \frac{905^1}{7.24 + 0.16} = 122 \text{ Krad}$$

[1] L.D.$_{50}$ = 905 (Read from Figure 1).

Cumulative values shown in columns *m* and *n* are based on two assumptions: first, that any tube not showing survivors at a given dose would not show survivors at a greater dose, and, second, that any tube showing survivors at a given dose would show survivors at a lesser dose. Values in column *m* for each dose were obtained by adding upward the number of positive tubes starting with the greatest dose and proceeding to the least. Cumulative values in column *n* for each dose were obtained by adding downward the number of negative tubes starting with the least dose and proceeding to the greatest dose. An equation for probability of sterility at any irradiation dose,

$$P = \frac{n + 1}{m + n + 2}^{c}.$$

was used to compute the probability (Column P). Plotting on arithmetic probability paper the dose against the probability of sterility yields a straight line fitted to the points. The dose read from the curve representing P = 0.50 is called the L.D.$_{50}$ point. It corresponds with a dose at which 50% of the tubes tested will be sterile. According to the

[c] LaPlace law of succession equation.

studies of Halvorson and Ziegler *(11)*, when 50% of such a series of tubes is sterile, the population is most likely to contain 0.69 surviving organisms per tube. Figure 1 shows probability of survival plotted against dose from the data in Table 1. The value of D_D was calculated according to the following equation:

$$D_D = \frac{L.D._{.80}}{\log N_o - \log 0.69} \text{ or } \frac{L.D._{.50}}{\log N_o + 0.16}$$

where N_o is the initial number of organisms per tube It is important that the spacing of doses should be so selected that partial survival points occur on both sides of the $L.D._{.80}$ point.

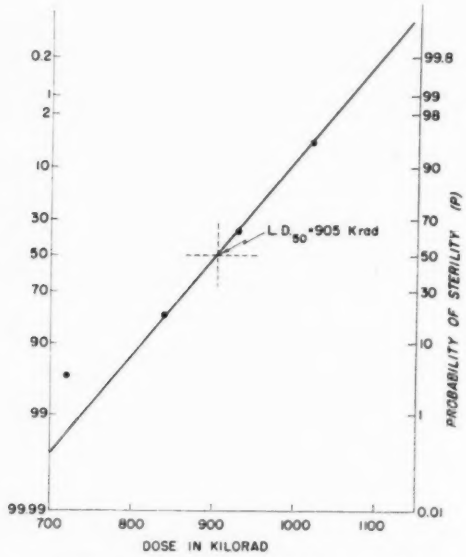

Figure 1. Arithmetic probability curve.

A comparison of the D_D values using $L.D._{.80}$ points obtained by the probability method and by the rigorous but tedious probit method of Finney *(6)* resulted in an average difference of 0.83 krad. The range of deviations was from −0.9 to +2.4 krad. Compared with the inherent errors in dosimetry, these small differences are regarded as unimportant.

Construction of survival curve. Since an exponential survival curve ploted on semi-log paper is linear, it may be defined in terms of a point and a slope. The slope is D_D. The point may be chosen as the log percent survival at the experimental sterilizing dose D_S, or the minimum dose which produced no positive tubes out of 10.

The percent destruction at the D_S point is based upon the following considerations. At a sublethal dose when 1 out of 10 tubes shows growth, it is assumed that the spore concentration is at least 0.1 per tube. When none out of 10 tubes shows growth at the lethal dose, the number of survivors remaining is less than 0.1 per tube. Arbitrarily choosing a midpoint between 0.1 and 0, a value of 0.05 per tube was adopted as a practical approximation for treating these results. Thus the percent survival at the destruction point was calculated as $\frac{0.05}{N_o} \times 100$, which locates the curve in the plane.

RESULTS AND DISCUSSION

Experiments to standardize the technics of the multiple tube probability method were carried out prior to a study of the radiosensitivity characteristics of *B. coagulans*. These studies were designed to clarify 3 problems:

a. Effect of geometric location of spore suspensions in radiation field on recovery of irradiated spores. Since there are 12 locations within the radiation rack at which sample urns may be placed, and 5 sites within each urn which could retain No. 2 cans, a maximum of 60 sites are possible. In early trials tubes containing spore suspensions were flame-sealed and were mounted at the bottom of water-filled cans. These cans were then placed at randomly selected locations in the radiation source in the rack. Table 2 shows such an experiment with 8 radiation sites in use. It will be noted that complete destruction resulted when an apparent dose of 650 krad

TABLE 2

Pattern of dose-survival data for B. coagulans spores as influenced by geometry of irradiation source

Incubation period of irradiated spores (*weeks*)		No. of tubes out of 10 showing survivors in various geometric locations							
	Site[1]	A-2(3)	C-2(5T)	B-1(3)	B-3(2)	C-1(4)	C-3(2)	B-2(3)	C-1(2)
	Dose rate *Krad/min*	8.90	10.40	9.00	21.10	17.15	19.17	18.10	17.15
	Total dose *Krad*	560	650	740	840	930	1020	1120	1210
1		9	0	1	2	0	0	0	0
2		10	0	2	8	0	1	0	0
3		10	0	5	8	0	1	0	0
4		10	0	5	9	0	1	0	0
5		10	0	5	9	0	1	0	0
6		10	0	5	9	0	1	0	0

[1] Rack and urn position, as designated in reference 2.

was supplied; yet half or more of the tubes showed bacterial survival when exposed to higher doses, as 740 and 840 krad, respectively. The mortality data do not follow the expected orderly progression of spore destruction with increasing dosage. It was learned that the glass cell dosimeters were conventionally mounted in the center of the corresponding cans. This meant that the center of the spore suspensions was well below the center of the dosimeters, and that the spotty pattern of kill was probably due to nonuniform dose distribution in the radiation field. When the geometry of the samples was corrected by elevating the spore suspensions to a level in the can corresponding with the midplane of the dosimeter, all subsequent experiments gave regular progression of spore destruction with increasing dose (Table 3). Experience has indicated that the uniformity of the radiation fields in the 3 central regions of the urn are quite satisfactory for precision dosimetry and mortality data.

b. Effect of incubation time on recovery of irradiated spores. Problems of dormancy in recovering cultures from heated spores have long been recognized *(5)*; hence the analogous situation with irradiated spores was explored. The importance of prolonged incubation which must be employed

TABLE 3

Influence of incubation time on radioresistance data for B. coagulans spores

Incubation period of irradiated spores (*weeks*)	No. of tubes out of 10 showing survivors at indicated irradiation dose[1]						
	560	650	740	840	930	1020	1120
1	10	10	9	4	2	0	0
2	10	10	10	6	3	0	0
3	10	10	10	6	4	0	0
4	10	10	10	8	4	0	0
5	10	10	10	8	4	0	0
6	10	10	10	8	4	0	0

[1] Irradiated in air at 17.16 Krad/min; $N_0 = 1.73 \times 10^7$ spores/ml in phosphate buffer pH 7.0; $D_D = 122$ Krad.

for overcoming dormancy of irradiated spores is illustrated in Tables 2 and 3. These results are representative of the great majority of the experiments performed. In no case has there been maximum recovery of spores in less than 3 weeks. In some instances 5 weeks of incubation were required for the maximum number of tubes showing germination and growth within the arbitrarily selected 6-week period. In contrast, it has been common practice for other investigators, who have studied *B. coagulans*, to enumerate irradiation survivors on agar plates after only 2 to 3 days of incubation.

c. Effect of time of addition of medium after irradiation on radioresistance of spores. If samples are irradiated at a gamma ray source and then transported to a distant bacteriological laboratory, it is possible that the time lag may change the viability of radiation-damaged spores. Such spores remaining in contact with the irradiated buffer solution might conceivably recover if nutrients were added shortly after irradiation. Another hypothesis was considered, namely, that toxic products might be formed during irradiation and that the damaged survivors on prolonged contact could succumb to the lethal action of such products.

Three replicate series of tubes containing spores suspended in phosphate buffer were irradiated and stored at 4 to 5° C. Each of the sets was then cultured, respectively, 0.5 hr, 24 hr, and 7 days after irradiation to simulate shipping intervals which might prevail. The findings are shown in Table 4. While the average D_D values appear to indicate a slight, consistent decrease with longer storage time, the small individual differences in radioresistance are within the experimental error of the method, since (a) the two extreme results vary within 10% of one another, and (b) the average D_S values show little or no variation. These results confirm the

TABLE 4

Effect of adding germination medium to irradiated B. coagulans spores at various time intervals. Spores suspended in phosphate buffer pH 7.0

Medium added after irradiation (*days*)	D_D value Krad			D_S value Krad		
	Expt. 1	Expt. 2	Ave.	Expt. 1	Expt. 2	Ave.
0	118	126	122	840	1020	930
1	121	113	117	1020	930	975
7	110	111	111	930	930	930

conclusions of Morgan and Reed *(13)* who, employing F.S. No. 1518 in pea puree, showed no consistent change in the recoverable number of gamma-irradiated spores during 4° C storage for periods as long as 8 weeks.

Having standardized the multiple tube probability method by eliminating some of the sources of error in technic, measurements of radiosensitivity as affected by gaseous environment, dose rate, initial spore concentrations, and protective substrates were next investigated.

Effects of gaseous environment and dose rate on radiation resistance in phosphate buffer. Approximately 400 tubes were filled with a phosphate buffered spore suspension. Half were canned under air and the remainder under nitrogen gas. The oxygen concentrations in the 2 atmospheres were about 21% and 2%, respectively. Also included in the experimental design was a comparison between a high and a low level irradiation dose rate, the high level selected being double the low intensity. For three replicate experimental runs conducted several months apart, the high level intensity varied in range from 20.6 to 34.6 kilorads per minute.

TABLE 5

Influence of gaseous environment on radioresistance of B. coagulans spores in phosphate buffer pH 7.0

Atmosphere	Dose rate level	D_D value[1] Krad	D_S value[1] Krad
Air	High[2]	129	1030
Air	Low	129	960
		Average: 129	1000
Nitrogen	High	154	1200
Nitrogen	Low	166	1270
		Average: 160	1240

[1] Mean value of 3 experiments.
[2] High level = ca. 2 x low level. High/low dose rates for 3 paired experiments:
Air .....20.6/10.4.....29.0/15.0.....34.6/17.2 (Krad per min)
Nitrogen.....20.8/10.6.....26.0/13.2.....33.1/14.6 (Krad per min)

Data in Table 5 show that the radioresistance of these spores was appreciably greater in tubes filled with nitrogen than with air. A lowering of oxygen concentration under these conditions resulted in a 24% increase of the D_D value, equivalent to a difference of 31,000 rads. This observation is at variance with the finding of Proctor and coworkers *(8, 9, 14, 15)*. Working with saline suspensions of an undesignated strain of *B. coagulans* spores, they reported no difference in resistance to gamma rays in air, oxygen or nitrogen.

Within the limited range of dose rates employed in these experiments, it does not appear that radiosensitivity was dependent on dose rate (Table 5). Doubling the intensity of gamma radiation had no influence on either the D_D or D_S values, regardless of the atmosphere used. Results obtained here with the multiple tube probability method are in accord with the earlier observations by a number of investigators *(3, 4, 10, 12, 13, 16, 20)* showing dose rate independence for the destruction of various bacteria by either gamma or cathode rays.

Effect of protective substrates on radioresistance in air and nitrogen. A series of experiments was carried out to determine the extent of radia-

TABLE 6

Influence of gaseous environment on radioresistance of B. coagulans in protein-fortified skim milk

Atmosphere	D_D value Krad	D_S value Krad
Air	198	1670
Air	182	1490
	Average: 190	1580
Nitrogen	214	1670
Nitrogen	217	1580
	Average: 216	1630

tion protection afforded by an aqueous substrate rich in organic matter and to determine whether nitrogen gas continued to exert its protective role in this medium. Protein-fortified skim milk was used as the suspending medium for the spores. For each experimental run about 300 tubes, containing such spore suspensions, were packed in cans. Half of the samples were air-sealed; the remainder were nitrogen-sealed, then irradiated. Results shown in Table 6 are compared graphically in Figure 2 with corresponding data for phosphate buffer.

The "substrate effect," as measured by the difference in D_D values between milk and buffer, was virtually the same for both gas packs; viz., 56 krad for nitrogen and 61 krad for air. It was also observed that the "oxygen effect," that is, the difference in D_D values between nitrogen gas

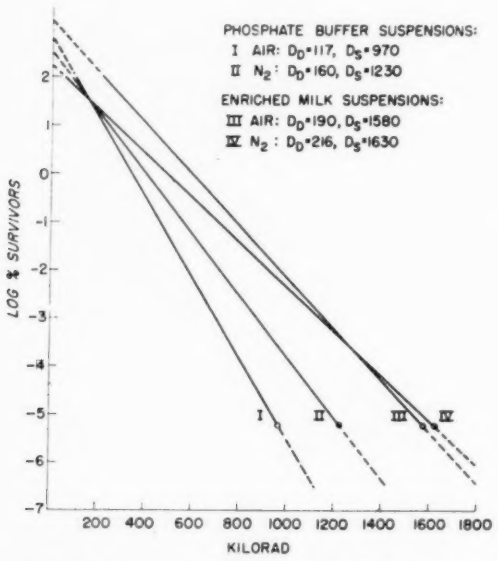

Figure 2. Effect of protective substrate and oxygen tension on resistance of B. coagulans spores to gamma rays. Approx. 1 x 10^6 spores per tube.

and air packs, was similar for both substrates; viz., 26 and 31 krad for milk and buffer media, respectively. It thus appears that the protective influence of nitrogen and of organic matter in the suspending menstruum were independent and additive factors.

Effect of spore concentration on D_D and D_S values. The D_D calculation is based on the premise that a majority of the species of bacteria undergo exponential destruction when exposed to ionizing radiations and thus possess straight line survival curves. Therefore, the D_D value, which represents the slope of the line, should remain independent of the initial number of organisms irradiated. To test this concept with the multiple tube probability method, a series of experiments was conducted employing spore concentrations of 10^4, 10^6 and 10^8 per tube. Data for phosphate buffer suspensions irradiated in air are presented in Table 7.

TABLE 7

Influence of initial spore populations on the D_D and D_S values of Bacillus coagulans in phosphate buffer pH 7.0

Initial spore concentration per tube	No. of replicate experiments	Average D_D value Krad	Average D_S value Krad
10^4	2	110	605
10^6	12	117.4	970
10^8	2	121	1300

The D_S values varied directly with the initial spore populations used, whereas values of D_D remained substantially constant. Differences in D_D values were well within the error limits of the method. Statistical analysis of the D_D values for the set of 12 replicate runs in Table 7 yielded a standard deviation of 5.25 krad. The 95% confidence limits for D_D values were 117.4 ± 11.55 krad. Individual D_D values for the set of 12 ranged between 109.5 and 125.7 krad. An apparent logarithmic order of death by gamma radiation was therefore observed for *B. coagulans* and is in agreement with the simple target theory.

Application of D_D values to irradiation preservation. The usefulness of D_D values lies in the computation of "sterilizing" doses. As an aid in the establishment of a food sterilization treatment, for example, it is necessary to know the appropriate D_D value and the initial number of organisms present in the food at the time of treatment. A sterilization process can thus be calculated to any degree of "sterility." Since D_D is the dose required to eliminate 90% of these contaminants, it follows that a $2 \times D_D$ dose will destroy 99% of the original load and an $8 \times D_D$ treatment, for example, will reduce the population by 99.999999%. In the experiments reported in Table 7 where each of the 10 replicate tubes contained about 10^6 spores, the D_S was actually $8.7 \times D_D$, or $D_S/D_D = 8.7$ log cycles of destruction. This means that the spore concentration had been reduced somewhat below 0.01 per tube in a 10-tube series. The ratio of D_S/D_D can therefore serve as a rough guide in the evaluation and selection of sterilizing doses. In the food canning industry, where a somewhat analogous situation occurs, thermal treatments equivalent to a 12 log cycle reduction

in population are commonly regarded as adequate for the safety of the thermally processed food.

SUMMARY

A multiple tube probability method for the determination of bacterial sensitivity to ionizing radiations is described, and its advantages are discussed based on studies of *Bacillus coagulans* spore resistance to gamma radiation.

Factors affecting the radiosensitivity of *B. coagulans* spores included gaseous environment during irradiation, protective nature of the medium in which the spores were irradiated, and the initial concentration of the spores. D_D values were 24% greater when phosphate buffer suspensions were irradiated in the presence of nitrogen gas instead of air. The resistance in protein-fortified skim milk medium was 43% higher than in phosphate buffer when the gaseous environment for each substrate was nitrogen. The magnitudes of the "oxygen effect" in buffer and milk, and the "substrate effect" in air and nitrogen were substantially the same; the substrate effect was twice as pronounced as the oxygen effect. A logarithmic order of death was indicated since it was found that wide variations in the initial spore concentrations (10^4, 10^6, and 10^8 spores per tube) did not change the D_D value while D_8 increased regularly with increases in spore population.

Acknowledgment

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LIPOLYTIC ACTIVITY OF MICROORGANISMS AT LOW AND INTERMEDIATE TEMPERATURES. I. ACTION OF *PSEUDOMONAS FLUORESCENS* ON LARD[a]

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The importance of lipolytic activity of microorganisms in meats, meat products, and dairy products held at low temperatures has been recognized for many years *(5, 6, 7, 9)*. Although there have been numerous investigations on various factors affecting the production of lipase by microorganisms, only a few have been concerned with the effects of temperature. Nashif and Nelson *(10)* found that *Pseudomonas fragi* had an optimum temperature around 15° C for lipase production and that several other Gram-negative bacteria produced maximum quantities of lipase around 20° C even though the optimum temperature for activity of the lipases was near 40° C *(12)*. Greene and Jezeski *(5)* reported a close correlation between total bacterial counts and onset of lipolytic activity by a *Pseudomonas* species over a temperature range of 0° to 30° C.

Balls, Matlack, and Tucker *(1)*, in studies with pancreatic lipase, found that the hydrolysis of the higher, but not the lower, saturated triglycerides was dependent upon temperature. When the pancreatic lipase was allowed to act for 72 hours at 0° C, the percentage hydrolysis of each triglyceride was as follows: C_4, 57%; C_6, 75%; C_8, 95%; C_{14}, 4%; C_{16}, 2%; C_{18}, 3%. However, at 40° C for 3 hours, the percentage hydrolysis of each triglyceride was: C_4, 60%; C_6, 76%; C_8, 96%; C_{14}, 99%; C_{16}, 97%; C_{18}, 87%.

In the above microbial investigations in which the effect of temperature was studied *(5, 11)*, the substrates were coconut oil and butterfat. Both of these lipids contain appreciable amounts of the short-chained fatty acids. The fat of pork and beef contains no short-chained fatty acids; yet their deterioration at low temperatures is of considerable economic importance. An investigation was initiated, therefore, to study the effect of temperature on the lipolytic activities of psychrophilic bacteria on animal fats. For such an investigation it was necessary to determine the best method for preparation of emulsions and to consider temperature and other factors involved. This paper is a report on the methods of study and the results of the action of a strain of *Pseudomonas fluorescens* on lard.

EXPERIMENTAL METHODS

The lard used was rendered in an open kettle according to standard practice without the addition of an antioxidant. It was packaged in 1-lb vacuum-packed cans and stored at −30° C until used in emulsions. Several relatively simple methods for preparing emulsions were tried. These were emulsification in a hand homogenizer with various

[a] Presented at the Nineteenth Annual Meeting of the Institute of Food Technologists, Philadelphia, Pennsylvania, May 18, 1959.

[b] Deceased.

emulsifying agents and the use of a Waring blender or Omni-Mixer [b] for the emulsification of lard-emulsifier mixtures. None of these procedures gave stable emulsions for prolonged periods of storage. The most satisfactory stock emulsions were prepared in a manner similar to that described by Goldman and Rayman *(4)*. In this procedure, preliminary emulsions of the lard (20% concentration), emulsifier (Astec 4135 [c], 2% concentration), and water were made at 57° C (± 3° C) in a Waring blender and then homogenized in a Manton-Gaulin homogenizer. A 2-liter dispensing funnel served as a reservoir on the intake side of the homogenizer and the liquid from the discharge side flowed into it to permit recycling of the emulsion. The reservoir was covered and a stream of nitrogen was continuously introduced into this closed, recirculating system. The temperature of the liquid was maintained at 55° to 60° C during homogenization by directing a stream of cold water over the homogenizer block and discharge pipe. The emulsion was recycled for 25 min at a pressure of 3500 psig. After homogenization it was placed in screw-capped bottles and autoclaved at 121° C for 15 min. When sterilization was completed, nitrogen was introduced into the autoclave as the pressure dropped, thus filling the head space of the bottles with nitrogen. The caps were screwed on tightly and the bottles stored at 3° to 5° C until used. Nearly all of the globules in these emulsions were less than one micron in diameter, and the emulsions were stable for several months. Peroxide values on these emulsions ranged from 1 to 7 and remained practically unchanged during storage.

The bacterium used in this investigation, *Pseudomonas fluorescens*, ATCC 11251, had been carried in stock culture for several years. This culture was moderately lipolytic and would grow at a temperature as low as −4° C.

In one series of experiments, the fat was present in the growth medium (initial pH 7.0) and the titratable acidity was determined at intervals from this culture. Uninoculated flasks incubated under identical conditions served as controls and the titratable acidity of the controls was subtracted from those of the inoculated flasks to give the net acidities shown in the data.

In the other series, the culture was grown in 1% peptone broth containing 0.05 M phosphate (initial pH 7.0 except in trials involving effect of pH) and the lipolytic activity of the culture supernatant determined at intervals. The supernatant was assayed as follows: Ten ml of supernatant containing the enzyme was added to a flask containing 5.0 ml of a 20% lard emulsion, 10.0 ml of 0.25 M phosphate buffer (0.25 M KH_2PO_4 adjusted to pH 7.0 with 0.25 M Na_2HPO_4), 1.0 ml of 1.8% formaldehyde (final concentration of 1:3000) and water to make a total volume of 50 ml. These flasks were incubated at 30° C for 24 hr. Assay medium was adjusted to pH 7.0 on the basis of preliminary work which showed that the optimum pH for activity of the enzyme ranged from 6.5 to 7.5. A similar activity range had been shown for lipase from other species of *Pseudomonas (10)*, although Lubert, Smith, and Thornton *(8)* found an optimum pH of 8–9 for a strain of *P. fluorescens*. Control flasks containing equivalent amounts of supernatant heated to 90°–98° C for 10 min were prepared in a similar manner. The titratable acidities of the control flasks were subtracted from those of the inoculated flasks and the differences in these acidities at zero and 24 hr are the net values shown in the data. It was found that the difference in the zero-hour and 24-hr titratable acidities of the inoculated flasks gave a net acidity very close to that in which the heated control flask was taken into account, and it could have been used without affecting the results.

The titratable acidity of all samples was determined as follows: A 10-g aliquot was weighed into a Mojonnier fat-extraction flask and acidified with 25% H_2SO_4 to approximately pH 2.5. Ten ml of ethyl alcohol were added and the mixture shaken thoroughly. Samples from flasks in which the organisms were grown in the presence of lard were extracted three times with 10 ml of petroleum ether. Samples from flasks containing culture supernatant and buffered lard substrate were extracted twice with 20 ml of a 1:1 mixture of ethyl ether and petroleum ether. Extracts were titrated with 0.02 N alcoholic NaOH using phenolphthalein as the indicator.

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[b] The mention of specific trade names throughout this paper does not constitute endorsement of the product used over comparable equipment and materials.

[c] Produced by American Lecithin Company, Inc., 57-01 32nd Avenue, Woodside, Long Island 77, New York.

Four media were compared to determine which would give maximum lipase production when lard in a final concentration of 5% was added. These media were: 1% peptone broth containing 0.05 M phosphate as suggested by Nashif and Nelson *(10)*, Goldman and Rayman's medium *(4)* in which yeast extract was the carbon and nitrogen source, a mineral salts-phosphate medium *(2)* in which ammonium sulfate was the nitrogen source, and BBL Trypticase Soy Broth. These media were inoculated with a 24-hr suspension of cells washed from a veal infusion agar slant.

To determine the effect of pH on production of the lipase, peptone broth without fat was prepared and inoculated as described above except that the initial pH was adjusted as desired. The pH in one experiment was maintained within 0.2 pH units of the initial value by adding after each determination the amount of acid necessary to return the flask contents to the desired pH.

All cultures and flasks except aerated cultures were incubated in thermostatically controlled water baths with a sensitivity of $\pm 0.2°$ C. Aerated cultures were incubated in a room held at 20° C ($\pm 1.0°$ C) on a reciprocating shaker operating at 180 cycles per min with a stroke of 2½ inches.

RESULTS

Results of the comparison of the 4 media for lipase production when lard was included in the growth medium are shown in Figure 1. The cultures grown in peptone-lard broth exhibited slightly higher activity than those in the Goldman-Rayman medium, although the difference was negligible for the first 10 days. This pattern of slightly greater lipolytic activity in peptone broth was repeated in cultures grown at 5° C and at 15° C. An additional experiment was carried out in which the culture was grown in these media without fat and the culture supernatant assayed for lipase activity. A similar slight superiority for the peptone broth was found in this comparison. On the basis of these findings the peptone broth was used throughout the remainder of the experiments.

The effect of pH on the production of lipase in a lard-free medium at 20° C is shown in Table 1. A similar series with the culture grown at 5° C is shown in Table 2. These data indicate that the optimum pH for production of the lipase is around 7.0, although there apparently was a broader optimum pH range at the lower temperature.

Nashif and Nelson *(11)* found more lipase activity in flask cultures of *P. fragi* than in cultures in test tubes; the differences in lipolytic activity, however, were less than the differences in total counts. Table 3 indicates that with the strain of *P. fluorescens* used

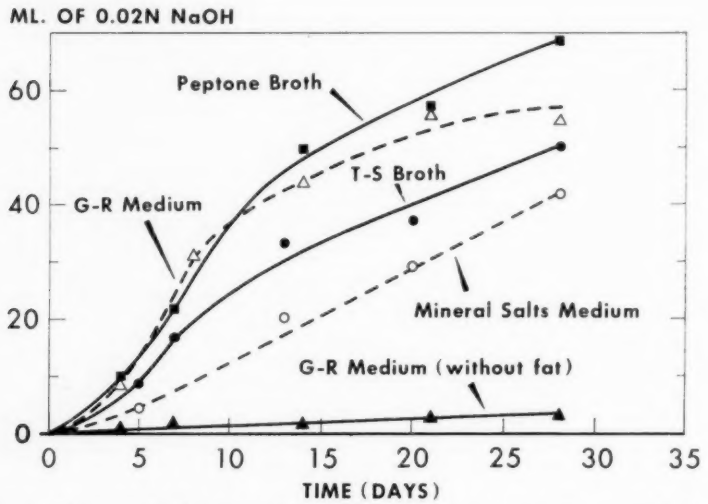

Figure 1. Lipase production at 20° C by P. fluorescens in various media containing lard.

TABLE 1

Effect of pH on production of lipase at 20° C by Pseudomonas fluorescens

pH range[1] or initial pH	pH and lipolytic activity of supernatant after:			
	3 days		4 days	
	pH	T. A.[2]	pH	T. A.
6.0 to 6.2		0.2		0.5
6.0	6.3	0.3	6.6	1.0
6.5 to 6.7		3.4		3.8
6.5	7.0	3.4	7.3	4.7
7.0 to 7.2		5.1		5.5
7.0	7.6	4.4	7.8	5.3
7.5 to 7.7		4.5		5.1
7.5	8.0	2.7	8.2	4.3

[1] The pH range shows the maximum change in pH during incubation.
[2] Titratable acidity in Ml, 0.02*N* NaOH.

TABLE 2

Effect of initial pH on the production of lipase at 5° C by Pseudomonas fluorescens

Initial pH	pH and lipolytic activity of supernatant after:					
	7 days		14 days		22 days	
	pH	T. A.[1]	pH	T. A.	pH	T. A.
6.5	6.6	0.2	6.8	3.7	7.1	3.9
7.0	7.1	0.0	7.5	3.0	7.8	3.9
7.5	7.7	0.0	7.9	1.9	8.2	3.3

[1] Titratable acidity in Ml, 0.02*N* NaOH.

TABLE 3

Effect of surface-volume ratio on lipase production and total count at 20° C

Volume of medium[1]	Net activity after 3 days[2]	Log of count after 3 days
50 ml	7.8	8.3
100 ml	2.2	7.9
200 ml	0.3	7.4
75 ml on shaker	2.0[3]	9.0[3]

[1] In a 300-ml Erlenmeyer flask, except 500-ml flask on shaker.
[2] Ml, 0.02*N* NaOH.
[3] These values were attained in 24 hr.

in this investigation the surface-volume ratio affects lipase production in some manner other than merely an effect on total count. Aeration is not the only variable involved since cultures grown on a shaker had high counts but low lipase production. Similar results were obtained in experiments in which the culture was grown with fat in the medium and the titratable acidity determined on aliquots removed at intervals.

Figure 2 shows the effect of temperature on the total count, lipase production, and lipase activity in a lard-peptone medium incubated at different temperatures. It is apparent from these data that the optimum temperature for lipase production is definitely lower than that for growth when either rate or total numbers are used as criteria for the latter. Further evidence that this effect was an actual decrease in production of enzyme rather than an accelerated destruction at the higher temperature is shown in Figure 3 for cells grown in fat-free medium. Cells grown at 31° C produced very little lipase during the entire incubation period whereas appreciable quantities were produced at 5° and 20° C.

Table 4 shows that the optimum temperature for activity of the enzyme is apparently unaffected by the temperature at which the enzyme was produced. Since incubation at 30° C had been selected for the first assays in this study to conform with the temperature used by other workers *(8, 10)*, the use of this temperature was continued even though greater activity occurred at 40° C.

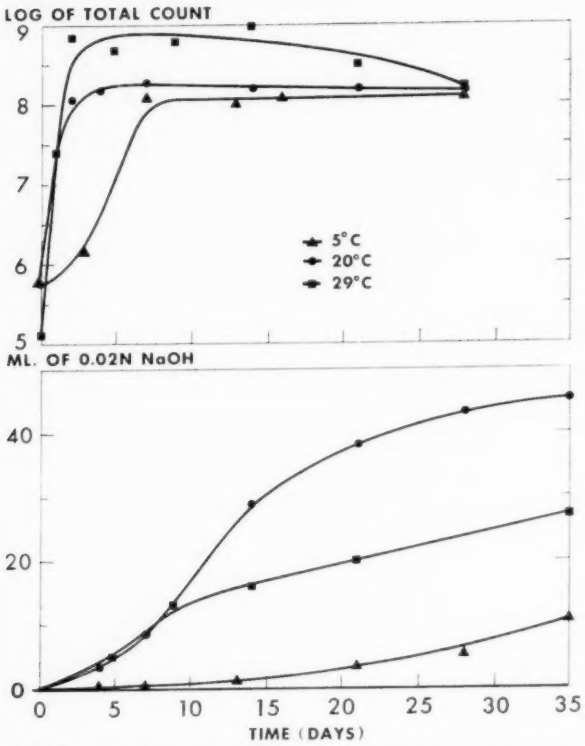

Figure 2. Effect of temperature on total count and lipolytic activity in lard-peptone medium.

TABLE 4

Effect of temperature on activity of lipase produced at different temperatu .s

Cultural conditions	Lipolytic activity[1] of culture supernatant at:				
	20° C	30° C	40° C	46° C	50° C
5 weeks at 1° C	1.2	2.7	4.6	4.0	
3 days at 20° C	2.1	3.4	5.8	5.1	3.2
2 days at 28° C	1.0	1.5	2.5	2.4	

[1] Ml, 0.02*N* NaOH.

A comparison of the 5° C curves and the 20° C curves in Figure 3 indicates that there was no depressing effect of temperature on lipase production at low temperatures as was shown for the higher temperatures. Once the lag period in lipase production was passed, the amount of lipase for a given number of cells grown at 5° C was at least equal to that for a similar count on the 20° C culture.

DISCUSSION

The effect of medium composition on lipase production substantiates the findings of others *(11, 13)* that microorganisms produce lipase in the absence of lipids and that an easily metabolized carbohydrate source retards lipase

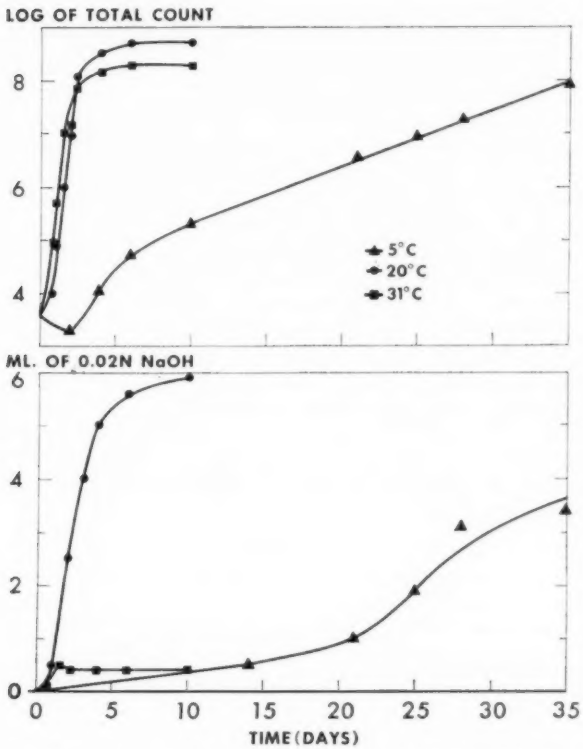

Figure 3. Effect of temperature on total count and lipase production in fat-free medium.

production. What might be interpreted as stimulation of lipase production by a simple nitrogen source probably should be interpreted as a non-specific response to a nutritionally inadequate medium. Because of the high yield of cells obtained with aerated cultures, this method of cultivation is often employed to give increased enzyme production. The data presented here, however, indicate that aeration retards production of lipase, although its production cannot be considered an anaerobic process since maximum yields were obtained in shallow, stationary layers of medium.

The depressing effect of increasing temperatures on the production of certain enzymes was noted several years ago by Gale *(3)*, who found that the production of amino acid decarboxylases by *Escherichia coli* was inhibited by raising the growth temperature. However, since the decarboxylases were strictly adaptive, it is of interest that a similar effect may occur with a constitutive enzyme such as lipase. Additional studies have been made on the effect of elevated incubation temperatures on the reactions obtained in several tests used for the differentiation of microorganisms. These results are being reported elsewhere.

Data in Tables 1 and 2 suggest that the lipase produced by cells grown at low temperatures may have a slightly lower pH optimum than that of cells grown at higher temperatures. More evidence is necessary to show this to be a significant difference, but it does indicate that an active lipase can be produced at low temperatures in acid foods.

The observation that the quantity of lipase produced per cell at low temperatures is at least as great as that produced at temperatures nearer the optimum for growth of the bacterium or activity of the enzyme has important implications in the storage of foods. Bacteria growing on food products held at low temperatures may not cause apparent fat deterioration as long as the food is held near 0° C; short periods of elevated temperature, however, could bring about definite lipolysis by the enzyme which had been formed at the lower temperature.

Although it may be reasonable to assume that any change in lipolytic activity with temperature is a quantitative rather than a qualitative response, the effect of triglyceride chain length shown by Balls, Matlack, and Tucker *(1)* may also be involved. This point is now being investigated. In any case, the data reported here indicate that it is possible to use lard, when it is properly emulsified, as a substrate in lipolytic investigations, even though the incubation temperature is low.

SUMMARY

A study has been made of the effect of medium composition surface-to-volume ratio, pH, and temperature on the production and activity of lipase by *Pseudomonas fluorescens* when lard was the substrate.

It was found that 1% peptone broth with an initial pH of 7.0 gave maximum lipase production. The yield of lipase per cell was much greater in shallow, stationary layers than in deeper layers, or in aerated cultures. The optimum pH for production of the enzyme was about 7.0, although it was produced down to pH 6.0.

At 20° C easily detectable levels of lipase were observed in 2–3 days, whereas production was very slight at 30° C, even though good cell yields were obtained. The amount of enzyme produced per cell at 5° C was equal to that produced at 20° C. The optimum temperature for the lipase was near 40° C and was not affected by the temperature at which the cells were grown.

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DEGREE OF MUSCULAR CONTRACTION AS A FACTOR IN TENDERNESS OF BEEF

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Connective tissue content *(6)*, histological arrangement of muscle fibres *(5, 6)*, storage history *(2)*, ionic balance and water retention *(1)* are all considered to be factors in the tenderness of beef. In a study on aging of beef it became apparent that the degree of contraction in which a muscle enters the state of rigor mortis is highly variable among the muscles of a carcass and might therefore be a significant factor in eating quality. A study, reported in detail elsewhere *(3)*, of striation patterns in different beef muscles by phase contrast and electron microscopy, has shown that four distinct types of fibril pattern exist, corresponding to definite sarcomere lengths. These are the familiar relaxed pattern, type I (3.7–2.4 mc), a well defined derivative pattern, type II (2.4–1.9 mc), a low contrast intermediate form, type III (1.8–1.5 mc) and the well known contraction pattern, type IV (1.5–0.7 mc). These pattern types and their length ranges were the same for the different muscles examined. The recognition of the fibril patterns therefore offers a convenient measure of the degree of contraction. The distribution of these four myofibril patterns is recorded for various muscles representing a complete range of tenderness.

EXPERIMENTAL

All experiments were carried out on prime ox. Most muscles were sampled in the boning room the day following slaughter and chilling. Part of each muscle was fixed in 10% formalin, while another part was left unfixed. In some cases muscles were excised immediately after slaughter, and samples were fixed within an hour of death and after standing in the cold overnight. Fixed samples were blended 5 min in water in a chilled blender, unfixed samples 1–2 min in 0.08*M* KCl. The suspensions of myofibrils were examined directly with a Zeiss Stand W phase contrast microscope. It was found that rigor muscle, whether fixed or unfixed, has the same distribution of fibril types. With the exception of *psoas* and *latissimus dorsi* excised at death, samples were obtained from at least two beasts. Good agreement was observed between animals. *Longissimus dorsi* excised at death (4 samples) showed some variability in rigor and a mean result is given.

For tasting tests 3 pairs of *psoas major* (6 judges) and 3 pairs of *psoas minor* (4 judges) were used. The front end of the muscle was freed on the hot carcass for about 7 inches, the flap was pinned up to relieve the weight, and the side was chilled with its uncut companion. Muscles were excised the following day, stored at 1° C and eaten at 3 days post mortem, after cooking in deep fat at 150° C to an internal temperature of 70° C.

RESULTS AND DISCUSSION

The distribution of pattern types in various muscles is shown in Table 1. The numerical values quoted are only very approximate visual estimates of the relative proportions. The tenderness and connective tissue ratings recorded in the table are those of Ramsbottom, Strandine, and Koonz *(6)*.

Variation in muscles chilled on the carcass. It may be seen that muscles cut from the carcass after rigor is complete fall into 2 categories, those

TABLE 1

Muscles	Treatment	Approximate proportions of fibril types (100% = 10) I 3.7–2.4	II 2.4–1.9	III 1.8–1.5	IV 1.5–0.7 (microns)	Results of Ramsbottom *et al* (6) Tenderness rating	Conn. tissue rating
Psoas major (front end)	ER	10	–	–	–	Very tender	5
	EFP	–	8	2	–		
	EPFR	–	1	8	1		
	CPER	1	8	1	–		
Psoas minor (front end)	ER	10	–	–	–	Very tender	–
	CPER	2	7	1	–		
Longissimus dorsi (near 6th rib)	ER	1	3	4	2	Slightly tender	6
	EFP	–	10	–	–		
	EPFR	–	2	4	4		
Tensor fasciae latae	ER	1	3	3	3	Medium	8
Semi-membranosus	ER	2	3	3	2	Slightly tough	8
Superficial pectoral	ER	10	–	–	–	Very tough	12
Cutaneous	ER	10	–	–	–	Very tough	13
Latissimus dorsi	ER	7	2	1	–	Very tough	14
	EFP	–	–	8	2		

ER = excised in rigor mortis.
EFP = excised and fixed prerigor (one hour after death).
EPFR = excised prerigor but fixed in rigor mortis.
CPER = cut (detached at front end only) prerigor but excised in rigor mortis.

which have only the relaxed[a] type I pattern *(psoas, cutaneous, superficial pectoral)* or predominantly this pattern *(latissimus dorsi)*, and those which show a broad distribution of patterns weighted towards intermediate types *(longissimus dorsi, tensor fasciae latae* and *semimembranosus)*. The final state of a muscle is probably determined by the strain imposed on it in hanging the carcass. For example the *psoas, latissimus dorsi,* and *cutaneous* are obviously under tension because of the unnatural position of the legs, whereas the *longissimus dorsi* and *semimembranosus* are much less so. The fact that the tenderest and toughest muscles are relaxed and those representing the middle range of tenderness are partially contracted does not support a relation between tenderness and relaxation. It must be remembered, however, that connective tissue content *(6)* is in increasing order reading down Table 1, so that the coarse structure and high connective tissue content of the last 3 muscles could be expected to outweigh the relaxed condition in regard to tenderness. In the medium class muscles both connective tissue and the degree of contraction could be significant contributors to toughness. The slight inferiority of the *longissimus dorsi* to the *psoas* for example could be in large part due to the partially contracted state of the former.

[a] The terms *relaxed* and *relaxation* are used in this paper in the sense of muscles with type I fibrils and may include muscles slightly contracted or stretched beyond the "resting length," which is not known.

The effect of removing restraint from the muscles. If muscles are excised or partially detached at death considerable shortening may occur as shown by the change in fibril pattern (Table 1). Generally a sudden shortening occurs at cutting and a further slow shortening takes place as rigor approaches.

The effect of shortening on the eating quality of psoas muscle. Tasting experiments were done on *psoas major* and *psoas minor* muscles which had been cut on the carcass and allowed to shorten by 20–30%. The fibrils of these muscles were mainly of type II, whereas the fibrils of the uncut muscles of corresponding sides used as controls were of type I. The meat was cooked 3 days after death, at which time rigor has resolved but aging affects are slight. Of the 6 pairs, the shortened muscles were in all cases judged the tougher. A single *psoas* excised at death shortened ca 50% to type III myofibrils and when cooked was very tough. It seems therefore, that in a good muscle where the effect of connective tissue is small, the state of contraction is a significant factor in tenderness. Contraction of the muscle progressively reduces the tenderness. This conclusion is supported by the work of Ramsbottom and Strandine *(7)* who found that beef boned 2 hours after slaughter and chilled was tougher than beef chilled on the carcass. Lowe and Stewart *(4)* found that cutting the breast muscle of poultry immediately after death caused a striking increase in toughness over the uncut muscle when roasted. The muscle they illustrate in Fig. 7 *(4)* has shortened by 20% on cutting.

Fortunately, the trade practice of chilling the whole carcass is a good one from the point of view of this factor in tenderness, as most muscles are prevented from shortening or restricted in shortening. In theory at least it should be possible to improve the quality of the *longissimus dorsi* for example by hanging the carcass in such a way that this muscle is stretched and prevented from shortening.

SUMMARY

The various muscles of the ox go into rigor in widely differing states of contraction as defined by the striation patterns of the myofibrils. The final state of a muscle appears to depend on the strain imposed on it in the hung carcass. It may be modified by cutting or excising the muscle. There is no correlation between the tenderness grading of the muscles and their contraction state on the carcass in rigor, but this could be due to the dominant effect of connective tissue. Tasting tests on *psoas* muscles which had been cut at death and allowed to shorten showed they were tougher than controls. It is concluded that relaxed muscles are tenderer than partly contracted muscles and that this effect may be significant in the grading of muscles of low connective tissue content.

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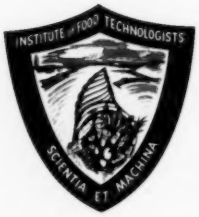
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